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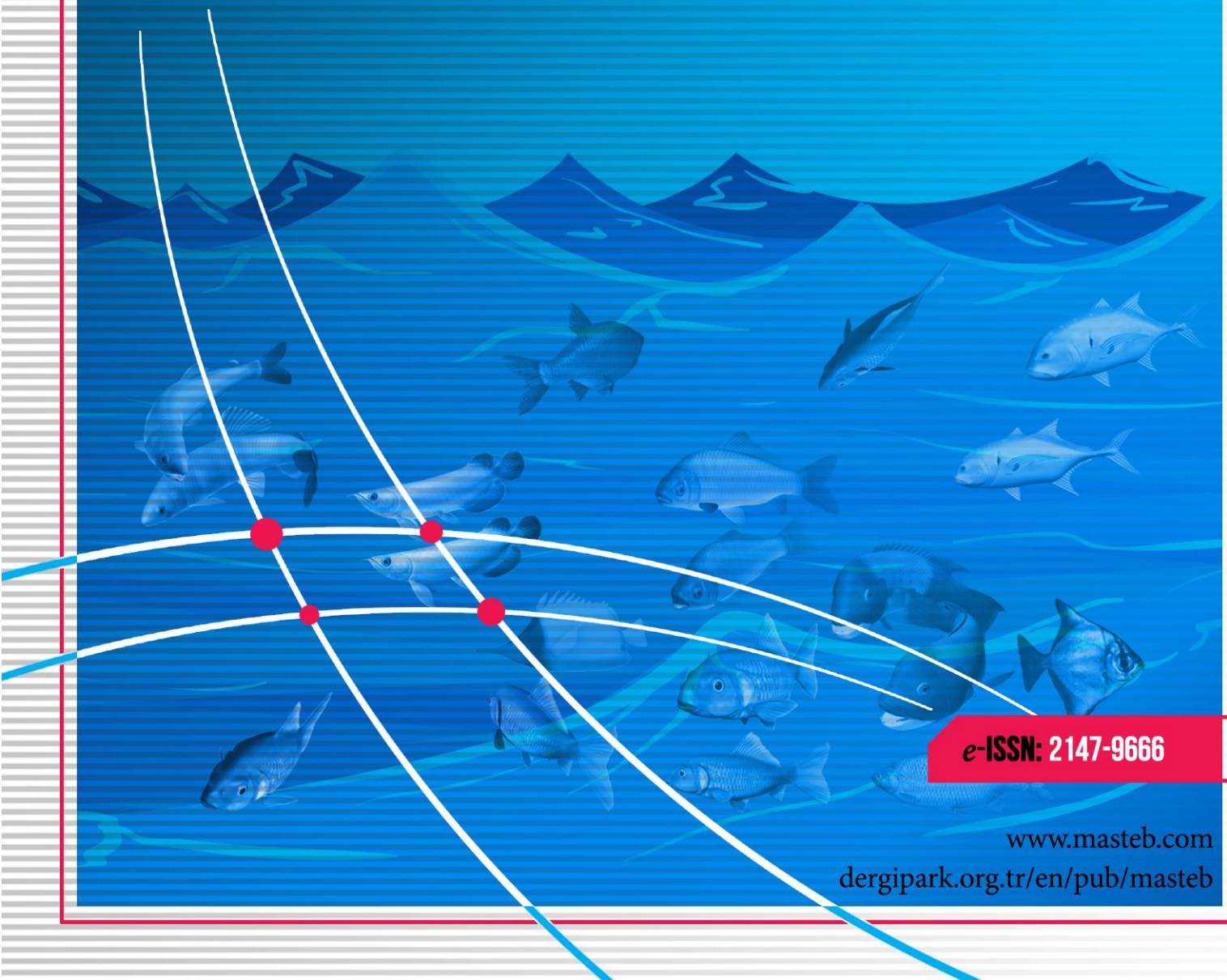

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RESEARCH ARTICLE

First record of gravid female American blue crab (*Callinectes sapidus* Rathbun 1986) from the Black Sea

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ABSTRACT

The occurrence of American blue crab, *Callinectes sapidus* Rathbun 1896, has been reported from the Black Sea, however, no gravid female has previously observed. Here, we record the first gravid female blue crab from the Black Sea. One individual of Atlantic blue crab was caught at 2 m depth using trammel net on 24th June 2020. The carapace width, carapace length, and the wet body weight of the crab were 200 mm, 81.03 mm, and 406.22 g, respectively. We also counted the eggs and measured the egg size. We further determined that the majority of the eggs were eyed, suggesting potential adaptation of the blue crabs to the Black Sea ecosystem.

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Introduction

Introductions of American blue crab have been reported from different localities on the coast of Europe including France, North Sea, Mediterranean Sea, Baltic Sea, Sea of Azov, and as well as Black Sea (Nehring, 2011). The species has reached a notable density in the Mediterranean and Aegean Seas (Holthuis & Gottlieb, 1955; Williams, 1974; Castriota et al., 2012), however, its existence in the Black Sea has been rare (Bulgurkov, 1968; Shaverdashvili & Ninua, 1975; Monin, 1984; Zaitsev, 1998; Bashtannyy et al., 2002; Diripasko et al., 2009; Khvorov, 2010; Pashkov et al., 2012; Aydın, 2017) (Figure 1), suggesting a low adaptation ability of the species to the low temperature values of the Black Sea (Nehring, 2011). This hypothesis might further be supported by the recent increase in the number of reports showing existence of the blue crabs from the Black Sea as the water temperature increases in the Black Sea during the "Mediterranization" process (Micu & Todorova, 2009). Besides the reports showing the occurrence of the American blue crabs, no report has yet indicated reproductive capability of this species in the Black Sea including gravid females.

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Material and Methods

We have collected a single gravid female of American blue crab from the mid of the Turkish coast of Black Sea (Figure 1). The individual was found at 2 m depth using a trammel net on 24th June 2020 in the Fatsa port, Ordu (41°02'45.81"'N and 37°29'31.83"'E). The individual was brought to the laboratory alive and was euthanized in ice. We then measured the carapace width and carapace length using a digital Vernier caliper to the nearest 0.01mm. We also measured the wet body weight of the crab. We collected three 1 g of subsamples from the clutch and counted the number of eggs. We finally measured the egg size (e.g. egg diameter) of 50 eyed eggs under a dissecting microscope.

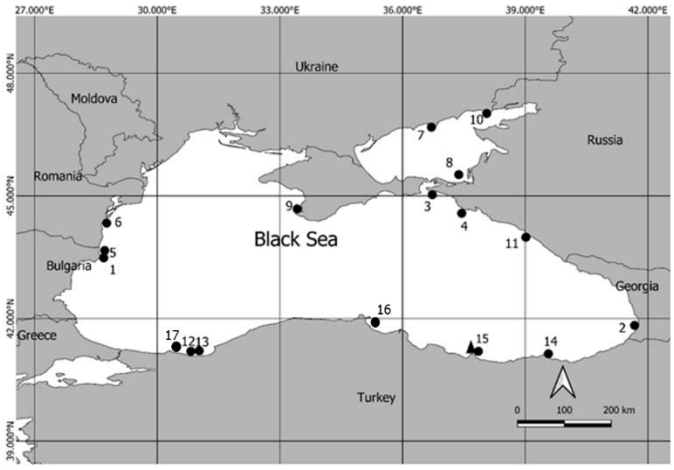

Figure 1. Map for the locations in the Black Sea where American blue crabs (*Callinectes sapidus*) have been reported. Locations are labeled chronologically from 1 to 15. The triangle indicates the site for the present study. Bulgurkov (1968)[1], Shaverdashvili & Ninua (1975)[2], Zaitsev (1998)[3,5], Monin (1984)[4], Bashtannyy et al. (2002)[6], Diripasko et al. (2009)[7,8,10], Khvorov (2010)[9], Pashkov et al. (2012)[11], Yağlıoğlu et al. (2014)[12, 13], Ak et al. (2015)[14], Aydın (2017)[15], Bilgin (2019)[16], Ceylan (2020)[17].

Results

The carapace width (including the longest spines) and length of the crab was 200 mm and 81.03 mm, respectively. The wet body weight was 406.22 g and the wet clutch weight was 33.84 g. The female was carrying a total of 1166879 eggs. The majority of the eggs were eyed (Figure 2) and the diameter of the eyed eggs varied between 261.7 µm and 309.5 µm (average=281±18.26 µm).

Discussion

This is the first record of a gravid female American blue crab in the Black Sea. Low water temperatures have been hypothesized to be the main causation for the unsuccessful establishment of the American blue crab in the Black Sea ecosystem (Nehring, 2011), since larval development and size at maturity are inversely related to the water temperatures (Hines et al., 2010) and no blue crab larvae can develop at water temperatures lower than 21°C (Hill et al., 1989). However, Black Sea ecosystem experiences strong consequences of the global changes, which is defined as the "Mediterranization" process, meaning that the Black Sea ecosystem is resembling to the Mediterranean character (Micu & Todorova, 2009). This transformation in the Black Sea ecosystem has led to increase in water temperatures and the salinity, which overlaps with the increased number of records showing introduced species to the Black Sea including the American blue crab. Our finding here shows that the increased water temperatures of the Black Sea allow blue crab eggs to develop and allow females to carry eyed eggs and potentially release them, thought we do not have any information on the survival of the larvae.

Aquatic species are often transported to new localities unintentionally via ballast waters of the ships. However, the species itself or life form of the species must be small enough to pass through the ballast water pump and intake ports, thus the species that are carried in the ballast water are often limited to phyto- and zooplankton, and other planktonic larvae belonging to a variety of macroinvertebrates and fish (Minchin & Gollasch, 2002). Therefore, it is reasonable to conclude that the gravid female that we collected was not transported to the Black Sea as an adult, and the coupling has happened in the Black Sea. Another potential explanation for the presence of the gravid female in the Black Sea was the migration of the blue crabs from

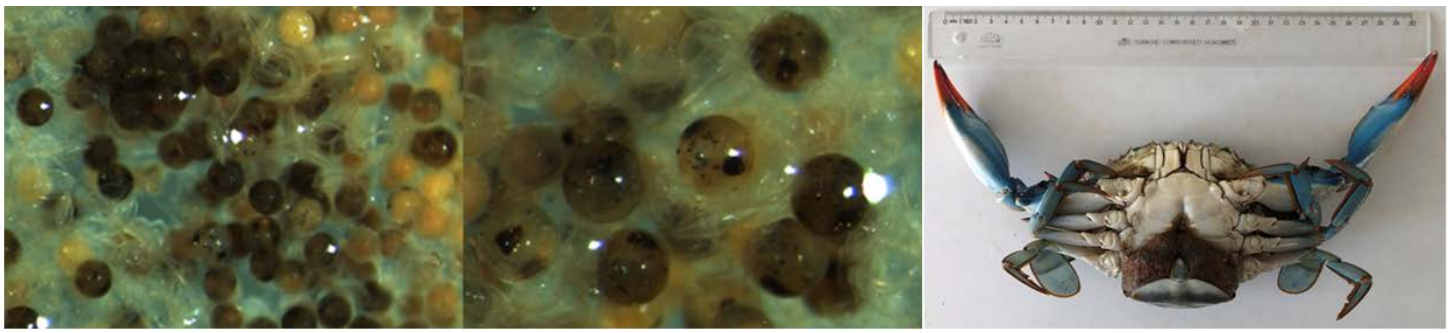

Figure 2. The views of the gravid female American blue crab (*Callinectes sapidus*) and the eyed eggs

the Aegean Sea (Öztürk et al., 2020), suggesting that coupling might happen in the Aegean Sea and the female individual migrated to the Black Sea. If this is correct for the blue crabs from the Black Sea, the idea of eggs and potentially larvae development of the blue crabs in the Black Sea should be true. In conclusion, our finding here has shown that American blue crabs are able to reproduce and develop eggs in the Black Sea.

Conclusion

Individuals of American blue crab (*Callinectes sapidus*) have long been reported from different coastal areas of the Black Sea. However, there has been a doubt about whether the species has a durable settlement in the Black Sea due to the lack of evidence for the reproduction. Our finding here shows that American blue crab has adapted to the Black Sea system by gaining the ability of reproduction.

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

For this study, all applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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RESEARCH ARTICLE

Length-weight relationships of four *Symphodus* species (Actinopterygii: Perciformes: Labridae) from Eastern Black Sea (Turkey)

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ABSTRACT

This study provides the length-weight relationships of four fish species that belong to the Labridae family from the Rize coast in the south-eastern Black Sea, Turkey; (*Symphodus ocellatus* (Linnaeus, 1758), *Symphodus cinereus* (Bonnaterre, 1788), *Symphodus tinca* (Linnaeus, 1758*)* and *Symphodus roissali* (Risso, 1810)). A total of 720 fish samples were collected with trammel net between June 2015 and May 2016. The sample sizes, minimum and maximum lengths and weights, length-weight relationships, parameters of *a* and *b*, ± 95% CI of *b*, r^2, growth type and statistical analyses of the relationship were determined. The *b* value estimates varied between 2.73 and 3.21. The r^2 value estimates varied between 0.76 (*S. ocellatus*) and 0.91 (other species).

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Introduction

The *Symphodus* species distributed in the East Atlantic, Black Sea and the Mediterranean to a depth of 1-50 m, on near cliffs and eel-grass beds (Whitehead et al., 1986). Some of its species may show sexual dimorphism and sex reversal (Whitehead et al., 1986). Some previous studies about the characterization of length-weight relationship (LWR) for fish species in the Black Sea, the coast of Turkey were discussed by Demirhan & Can, 2007; Kalaycı et al., 2007; Ak et al., 2009; Yankova et al., 2011; Ergüden et al., 2011; Özdemir & Duyar, 2013; Kasapoğlu & Düzgüneş, 2013; Satılmış et al., 2014; Gündoğdu et al., 2016; Çalık & Sağlam, 2017; Samsun et al., 2017; Türker & Bal, 2018; Yıldız et al., 2018). However, there is no data on the length-weight relationships of wrasse species in these studies. There are two studies on Lapin species in the Eastern Black Sea Region. Kalaycı et al. (2007) studied the meat productivity of the species, Kasapoğlu et al. (2016) studied some biological characteristics of the species *S. tinca*. There are some length-weight relationship studies conducted in other seas and involving wrasse species (Valle et al., 2003; Pallaoro & Jardas, 2003; Verdiell-Cubedo et al., 2006; Özaydın et al., 2007; İlkyaz et al, 2008; İlhan et al., 2008; Keskin & Gaygusuz, 2010; Gürkan

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et al., 2010; Bilge et al., 2014; Dimitriadis & Fournari-Konstantinidou, 2018).

This study aimed to provide data on the relationship between the length and weight of four wrasse fish species captured from the coastal waters of the Eastern Black Sea, Turkey. This research is the first study to determine the LWR of wrasse species in the Eastern Black Sea.

Material and Methods

Study Area and Fish Sampling

This study was carried out on four fish species belonging to the Labridae family, which were caught as discard fish during red mullet fishing in Rize region of the Eastern Black Sea between June 2015 and May 2016 (Figure 1). In this region, fishes were obtained monthly from the fishermen and they were transferred to the laboratory. The fishing was carried out at a depth of 10-40 m with trammel nets (20-24 mm in mesh size).

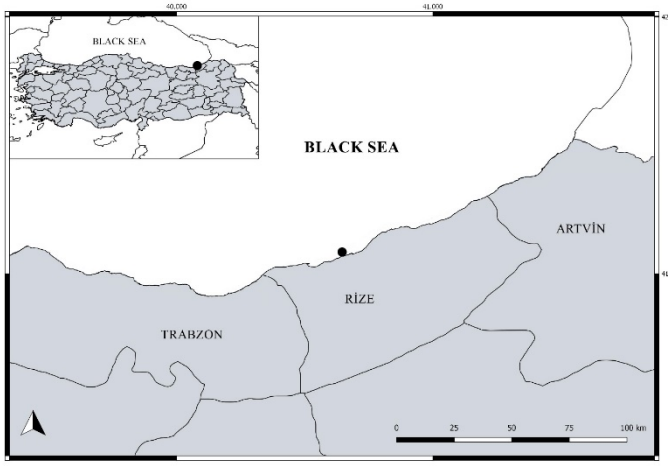

Figure 1. Study area

Length- Weight Relationship (LWR)

Fish species were identified at species level and verified with reference to the Fish Base (Froese & Pauly, 2017). The weight (W) was determined to the nearest 0.01 g and the total length (TL) was measured to the nearest 0.1 cm. The parameters *a* and *b* of relationships of the Equation (1) (Ricker, 1973, 1975) were estimated through logarithmic transformation (Equation 2);

$$W = aL^b \quad (1)$$

$$\log W = \log a + b \log TL \quad (2)$$

where *W* is weight (g), *TL* is total length (cm), *a* is the intercept and *b* is the slope of the linear regression. Parameters *a* and *b* were calculated by least-squares regression, as was the coefficient of determination (r^2). Additionally, 95% confidence limits of the parameter b were estimated. The growth type was identified according to Equation (3):

$$ts = \frac{b-3}{SE(b)} \quad (3)$$

where *ts* is a t-test value, *b* is a slope, and SE(*b*) is a standard error of the slope. According to t-test value of *b*, the growth type was determined as isometric (*b*=3), negative allometric (*b*<3), and positive allometric (*b*>3) (Dutta et al., 2012). All the statistical analyses were evaluated at a 5% significance level ($p<0.05$) (Zar, 1999).

Results

In this research, LWR for 4 species (*S. ocellatus* (n=384), *S. cinereus* (n=321), *S. tinca* (n=17) and *S. roissali* (n=18)) were examined (Figure 2). For all species, sample sizes, length ranges (minimum-maximum), parameters of length-weight relationships (*a* and *b*), 95% confidence intervals of *b* and the coefficient of determination (r^2) and growth types were given in Table 1.

The total length for *S. ocellatus* was between 6.9 and 16.4 cm (11.56 ± 0.050 cm) and the total weight was between 4.77 and 79.97 g (23.36 ± 0.35 g). The total length for *S. cinereus* was between 8.2 and 16.4 cm (12.46 ± 0.069 cm) and the total weight was between 8.51 and 77.49 g (35.28 ± 0.65 g). The total length for *S. tinca* was between 6.5 and 12.8 cm (11.56 ± 0.37 cm) and the total weight was between 3.87 and 36.07 g (20.86 ± 1.77 g). The total length for *S. roissali* 7.4 and 12.2 cm (11.56 ± 0.26 cm) and the total weight was between 6.11 and 28.51 g (21.04 ± 1.34 g). The most abundant species were *S. cinereus* (51.89 %), *S. ocellatus* (43.37 %), *S. roissali* (2.43 %) and *S. tinca* (2.31 %).

Table 1. Length-weight relationships of 4 fish species caught from Eastern Black Sea, Turkey

Species	N	$TL_{min-max}$	$W_{min.-max.}$	*a*	*b*	SE(*b*)	95% CL of b	r^2	t-test
S. ocellatus	321	6.9-16.4	4.77-79.97	0.028	2.73	0.0071	2.89-2.56	0.76	2,77
S. cinereus	384	8.2-16.4	8.51-77.49	0.010	3.21	0.0024	3.31-3.12	0.91	1,67
S. tinca	17	6.5-12.8	3.87-36.07	0.016	2.99	0.0561	3.49-2.49	0.91	0,37
S. roissali	18	7.4-12.2	6.11-28.51	0.014	3.06	0.0535	3.55-2.57	0.91	0,37

Note: *N*: sample size; *TL*: length type: *W*: weight; *min*: minimum; *max*: maximum; *CI*: Confidence interval; *a* and *b* relationship parameters; *SE(b)*: Standard error of b; *r*: Coefficient of determination.

Table 2. LWR parameters fish species estimated from other areas

Species	N	$L_{min-max}$	$W_{min.-max.}$	a	b	r	Region	Reference
S. ocellatus	216	4.7-9.7	-	0.009	3.22	0.96	Aegean	Özaydın et al., 2007
	328	4.7-9.2	1.10-10.56	0.009	3.19	0.97	Aegean	İlhan et al., 2008
	575	1.8-10.7	0.0093	0.010	3.08	0.97	Marmara	Keskin & Gaygusuz, 2010
	10	4.30-6.60	0.59-2.79	0.004	3.48	0.98	Aegean	Gürkan et al., 2010
	456	3-9	-	0.009	3.17	0.96	Mediterranean	Valle et al., 2003
	1922	1.4-18.5	0.01-81.69	0.01	3.2	0.98	Aegean	Altın et al., 2015
	274	4.6-9	-	0.010	3.13	0.95	Aegean	Bilge et al., 2014
S. cinereus	8	6.6-8.6	-	0.008	3.26	0.99	Aegean	İlkyaz et al., 2008
	20	4-7	-	0.011	3.07	0.95	Aegean	Özaydın et al., 2007
	58	22-106	-	0.011	3.11	0.99	Mediterranean	Verdiell-Cubedo et al., 2006
	92	4.5-10.1	2.19-25.35	0.023	3.03	0.86	Aegean	İlhan et al., 2008
	173	2.3-11.3	-	0.009	3.18	0.99	Marmara	Keskin & Gaygusuz, 2010
	4	5.90-7.60	2.9-6.03	0.007	3.24	0.96	Aegean	Gürkan et al., 2010
	665	3.6-15.4	-	0.010	3.13	0.97	Mediterranean	Valle et al., 2003
	61	4.7-9.7	-	0.005	3.51	0.91	Aegean	Bilge et al., 2014
	536	1.5-15.8	0.02-56.32	0.01	3.2	0.96	Aegean	Altın et al., 2015
S. tinca	89	6.7-23	-	0.018	2.91	0.98	Aegean	Özaydın et al., 2007
	277	6.7-24.3	4.28-185.16	0.018	2.91	0.98	Aegean	İlhan et al., 2008
	41	2.1-15.5	-	0.011	3.10	0.99	Marmara	Keskin & Gaygusuz, 2010
	10	4.70-10.50	1-12.09	0.013	2.89	0.96	Aegean	Gürkan et al., 2010
	56	11.4-30.4	-	0.026	2.79	0.97	Mediterranean	Valle et al., 2003
	1443	8.6-42.5	7.9-679.8	0.022	2.81	0.98	Adriatic	Pallaoro & Jardas, 2003
	83	12.4-25.3	30-205	0.026	2.76	0.97	Ionian sea	Dimitriadis & Fournari-Konstantinidou, 2018
	110	6.6-22	-	0.018	2.92	0.96	Aegean	Bilge et al., 2014
	60	11.6-25	22-186	0.019	2.84	0.99	Mediterranean	Miled-Fathalli et al., 2019
	27	3-18.5	0.23-77.22	0.010	3.30	0.99	Aegean	Altın et al., 2015
	248	10-26.8	-	0.010	3.04	0.97	Aegean	Karakulak et al., 2006
S. roissali	22	2.4-14.1	-	0.007	3.39	0.98	Marmara	Keskin & Gaygusuz, 2010
	120	-	-	0.035	2.67	0.95	Mediterranean	Gordoa et al., 2000

Regarding the type of growth, two species (*S. ocellatus*, and *S. tinca*) showed positive allometry, two species (*S. cinereus* and *S. rossali*) showed negative allometry. In the findings of this study, the "*a*" values ranged from 0.0010 to 0.028 while the "*b*" parameters varied between 2.73 and 3.21. The determination coefficients (r^2) ranged between 0.76 (for *S. ocellatus)* and 0.91 (for other species).

Discussion

In this research, 740 fish samples belonging to the Labridae family and to four species were caught and examined. Due to the lack of other studies in the region, the study was compared with some studies conducted in other seas.

While the b value for *S. ocellatus* ranged between 3.08 and 3.48 in other studies (Valle et al., 2003; Özaydın et al, 2007; İlhan et al, 2008; Keskin & Gaygusuz, 2010; Gürkan et al, 2010; Bilge et al., 2014; Altın et al., 2015). It was found to be 2.73 in our study. In addition, the r value for *S. ocellatus* was calculated as 0.76. This value is low, this may be related to the nutrition of individuals. The b value for *S. cinereus* ranged from 3.03 to 3.51 in other studies. In this study, the b value was determined to be 3.22, similar to other studies (Table 2). The b value for *S. tinca* was calculated as 2.99. While this value differs with the work of Keskin & Gaygusuz (2010), it is similar to other studies (Table 2). The b value for *S. roissali* was determined to be 3.06. While this value differs from the work of Gordo et al. (2000), it is similar to Keskin & Gaygusuz (2010).

The LWR is affected by some factors such as stomach fullness sex, preservation techniques, feeding habits, health, maturity, season, and habitat (Tesch, 1968), and annual differences in environmental conditions (Froese, 2006). In addition, differences in the period and size composition of the sampling might affect *b* values of the LWR (Table 2). Furthermore, some authors used the standard length (SL) while others used the total length (TL).

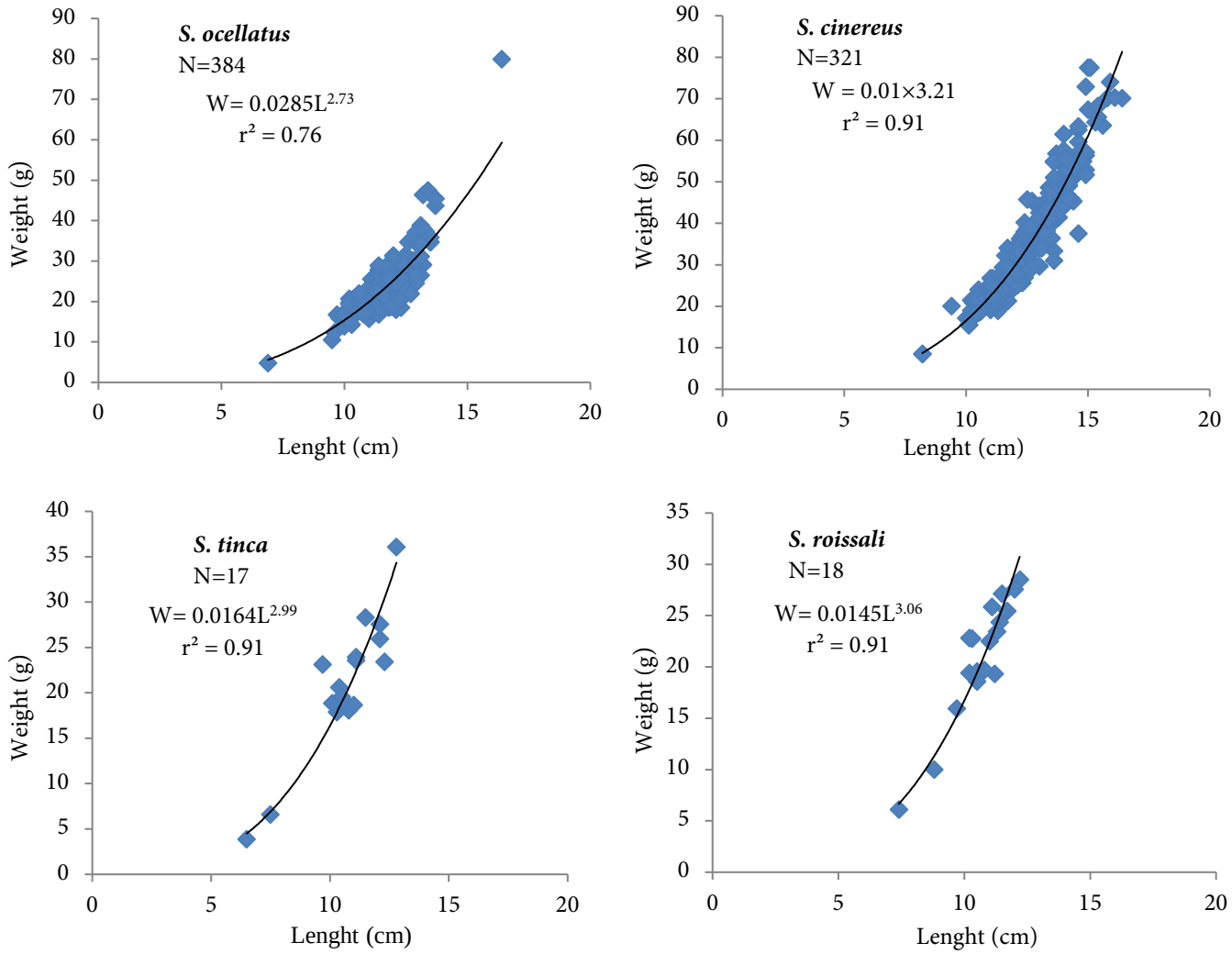

Figure 2. The curves of length-weight relationships for four *Symphodus* species from the Rize coast in the south-eastern Black Sea, Turkey

Indeed, the estimation of LWR is needed and important as it provides information on population conditions. It is also a widely applied approach in the study of the dynamics of exploited stocks and an effective tool used for basic research and management strategies in fisheries. Moreover, the parameters of length-weight relationships allow other authors to make comparisons between different populations of the same living species in similar or different ecosystems (Pauly, 1993; Petrakis & Stergiou, 1995; Gongalves et al., 1997).

Conclusion

With this study, new findings on the wrasse fish in the Black Sea basin were determined. The data related to the fish on the coast of Turkey will contribute to future works. In addition, these data can be used in the assessment of fish stocks of species that have not yet been exploited and have no economic value. Moreover, the increasing need for protein is important in terms of bringing these species to the economy and consuming them as human food. Further studies of this species are required to expand our knowledge of life cycles in the conditions of the Black Sea basin.

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Compliance with Ethical Standards

Conflict of Interest

The author confirms that no conflicts of interest exist and the funders had no role in study design, data collection, analysis, and decisions.

Ethical Approval

For this type of study, formal consent is not required.

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REVIEW PAPER

Ice-ice disease in commercially cultivated seaweeds *Kappaphycus* spp. and *Eucheuma* spp.: A review on the causes, occurrence, and control measures

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ABSTRACT

Kappaphycus spp. and *Eucheuma* spp. are two economically significant seaweed species cultivated globally due to their carrageenan content with numerous commercial applications. They are mainly cultivated in the Philippines, Indonesia, Malaysia, and Tanzania. The culture of these seaweeds also provides income sources for many coastal dwellers. In 2018, the total global production from these seaweeds was about 11 million tonnes. One of the primary problems that affect seaweed production is the incidence of ice-ice disease. In this article, we reviewed the reported scientific journals on the ice-ice disease of two commercially cultured seaweed species (*Kappaphycus* spp. and *Eucheuma* spp.), focusing mainly on causes, occurrence, and control measures. The ice-ice disease is caused by both abiotic and biotic factors manifested by the presence of white and soft parts in the infected seaweeds. The occurrence of this disease varies from species, places, and seasons. Control measures may include proper farm management, polyculture with other seaweeds, pre-soaking with antibacterial substances and nutrient enrichment before out-planting, and possibly using genetic engineering.

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Introduction

Commercial seaweed species like *Kappaphycus* spp. and *Eucheuma* spp. are significant seaweed species used as a carrageenan source, a phycocolloid, with various usage area in commercial applications as a thickening, gelling, and binding agents in food products and sauces, as well as in experimental medicine, pharmaceutical cosmetics, formulations, and

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industrial applications (Necas & Bartosikova, 2013; Loureiro et al., 2017a). Nearly 90% of the world's carrageenan comes from these seaweed species primarily cultured in Indonesia and the Philippines (Campbell & Hotchkiss, 2017). The annual global production of carrageenan is over 60,000 tons with a gross market value of US$ 626 million (Rhein-Knudsen et al., 2015). In many coastal tropical and sub-tropical countries, these seaweeds' farming is an essential livelihood source (Hayashi et al., 2017). The combined world production of these seaweeds in 2018 was around 11 million metric tons comprising 34% of the total seaweed production globally (FAO, 2020).

One of the main hurdles in the farming of these macroalgae is diseases, e.g., ice-ice disease and epiphyte infestation (Largo, 2002). These diseases developed due to increased farm size and culture practices intensification (Vairappan, 2006). These are responsible for many seaweed seedlings losses, influencing both biomass quantity and quality, which primarily affect marginalized coastal villages relying on seaweed farming as a sole income source (Loureiro et al., 2017b).

Ice-ice disease, in particular, is the most prevailing problem seen in the farming of eucheumatoids worldwide. This disease is caused primarily by stressful environmental conditions, as Largo et al. (1995a) demonstrated in laboratory experiments and likewise observed in the farm field (Arisandi & Farid, 2014). Pathogenic bacteria further degrade the diseased seaweeds lead to eventual thalli disintegration (Largo et al., 1995b). Marine-derived fungi also play an important role in ice-ice disease induction (Solis et al., 2010). This disease is characterized by the appearance of white and soft parts on the infected seaweeds' thalli.

Disease problems (ice-ice disease and epiphyte infestations) in eucheumatoid farming have resulted in shrinking culture stocks and decreased quality of carrageenan, resulting in low market value and income and job opportunities losses, especially for marginal seaweed farmers (Ward et al., 2020). In the Philippines, ice-ice disease occurrence and epiphyte infestations have a drastic effect, causing 15% *Kappaphycus* production losses between 2011 and 2013 (Cottier-Cook et al., 2016). In Zanzibar, Tanzania, these diseases and pests become a menace to the commercially cultivated eucheumatoids, affecting seaweed farmers (Largo et al., 2020). Ice-ice disease lowers the carrageenan quality of *Kappaphycus striatus* "sacol" strain; hence, removing the diseased portion is recommended before drying (Mendoza et al., 2002).

Only a few reviews on cultivated seaweeds' pests and diseases are available (Largo, 2002; Ward et al., 2020). No recent review articles are available that focus on ice-ice disease, particularly the causes, occurrence, and treatments. Thus, this paper reviewed all the reported papers on the ice-ice disease, its causes, occurrence, and control measures, focusing on the *Kappaphycus* and *Eucheuma* species commonly cultivated worldwide.

Methods

This study reviewed the available articles from the literature by searching keywords such as ice-ice disease, *Kappaphycus,* and *Eucheuma* in Scopus and Mendeley databases published from 1977 to 2021. Online and printed theses related to this study were also considered. Articles published beyond the time of writing this review were not included.

Occurrence of Ice-ice Disease

The occurrence of the ice-ice disease varies from place to place and season. In Lian Bay, Hainan Province, China, the outbreak pattern in *Kappaphycus* spp. mostly occur from May to August, and October is the month where the outbreak pattern is the same with epiphyte *Neosiphonia savatieri* (Pang et al., 2015). Similarly, the month of April, October, and December noted the highest ice-ice incidence of *Kappaphycus alvarezii* and *Eucheuma denticulatum* in Bais Bay, Negros Oriental and Zamboanga del Norte, Philippines, which ranged from about 52 to 56% (Tisera & Naguit, 2009). Furthermore, in Zamboanga City and Zamboanga del Sur, Philippines, the ice-ice disease occurrence of *K. alvarezii* ranged from about 22 to 40% observed during July to September (Alibon et al., 2019). Farmed *K. alvarezii* in Mannar Gulf and Palk Bay, Southern India, also experienced ice-ice occurrence in March and April (Arasamuthu & Edward, 2018). Even in Zanzibar, Tanzania, the development of ice-ice disease was tremendous during February – March (hot, dry season) and typically diminished during May – June (wet season) by up to 99% (Largo et al., 2020). A month of May was also recorded a high ice-ice disease incidence ranging from 70% to 87% in Calaguas Is., Camarines Norte, Philippines (Hurtado et al., 2006). In South Sulawesi, Indonesia, the ice-ice disease occurred in September – October (dry season) after the emergence of epiphytic infestation (Badraeni et al., 2020). The occurrence of this disease indicates that most of the mentioned months are associated with environmental factors such as elevated temperature and low salinity, which are separately discussed below.

Environmental Factors Causing Ice-Ice Diseases Development

Temperature

It has been demonstrated in the laboratory experiment of Largo et al. (1995a) that stressful environmental conditions are detrimental to cultivated *K. alvarezii*. In their temperature experiments, seaweeds incubated on the weekly interval in two sets of temperature regimes starting from 25°C up to 35°C and from 20°C down to 15°C in a stepwise manner and revealed that extreme temperatures (33°C–35°C) caused extensive whitening resulting to total impairment of the thalli, and these damaged tissues resemble the ice-ice infected branches in seaweed farms. The recent paper of Largo et al. (2020) indicated that intense temperature (29.5°C–35.5°C) in seaweed farms in Zanzibar, Tanzania is one of the prime triggers of ice-ice development and epiphyte infestation. Whitening of the ice-ice infected thalli in *K. alvarezii* affects its photosynthetic efficacy due to the significant reduction in photosynthetic pigment concentration leading to sluggish growth before ice-ice occurrence (Ganzon-Fortes et al., 1993). Similarly, high surface temperatures have been associated with ice-ice disease and other pests (fouling and epiphytes), which caused a substantial decline in seaweed production in Song Song Island, Tanzania (Msuya & Porter, 2014).

Salinity

Kappahycus and *Eucheuma* are true marine red seaweeds; hence, salinity fluctuations in the farms negatively affect cultured seaweeds, including the ice-ice disease development. Laboratory experiments of Largo et al. (1995a) showed *E. denticulatum* exposed to salinity 25‰ and 35‰ responded positively. In contrast, the salinity of 20‰ below within a week is considered deleterious, which led to the ice-ice ice disease development. The whitened parts have prominent similarities to those noted in the seaweed farms.

Light Intensity

The utmost significance of light to seaweeds is supplying the energy for photosynthesis and is considered the most important abiotic factor affecting seaweeds (Hurd et al., 2014). Light intensity has been suspected as one inducer in ice-ice disease development (Largo et al., 1995a). To elucidate the effects, the authors used an increasing light intensity beginning at 0, then elevated to 10, 20, 50, 100, and up to 125 mol photon m^{-2} s^{-1} at the seven-day interval and concluded that less than 50 mol photon m^{-2} s^{-1} light intensity led to ice-ice whitening. These conditions are comparable in the farms when the seaweeds are crowding due to overstocking of the branches in the lines and hindrance due to epiphytes presence, which may create an artificial effect that is lethal. Hence, ice-ice disease development in the farms is typically associated with epiphytes incidence (Trono & Ohno, 1989; Uyenco et al., 1977). In Zanzibar, Tanzania, seaweeds, farmed in shallow intertidal lagoons nearly in direct contact with seafloor bottom during low tides, received extreme levels of light intensity and temperature caused ice-ice disease outbreak (Largo et al., 2020).

Nutrients

Seaweeds require an extensive array of nutrients for growth. In the most natural environment, seaweed growth and yields are limited by the availability of two nutrients, nitrogen and phosphorus (Harrison & Hurd, 2001; Roleda & Hurd, 2019).

Kappaphycus cultivation depends chiefly on the sea's natural fertility (Luhan et al., 2015). Hence, extensive farming may result in the outbreak of ice-ice disease (Vairappan, 2006). Maryunus (2018) reported that nutrient deficiency is the primary factor triggering the ice-ice disease development, as shown by the relationship between nutrient deficiency (nitrogen and phosphorus) and the ice-ice disease outbreak.

Biological factors causing ice-ice disease

Pathogenic Marine Bacteria as Secondary Causative Agents

There are limited studies on the marine bacteria isolation and identification from ice-ice diseased seaweeds *Kappaphycus* and *Eucheuma* and undergoing into pathogenicity test. Table 1 summarizes the previously reported works on the associated and pathogenic bacteria isolated from the seaweeds infected with the ice-ice disease.

The mechanism of ice-ice disease-causing bacteria as secondary causative agents is still not fully clear and explored. Some studies (Largo et al., 1995b, 1998, 1999) have suggested that pathogenic bacteria *Vibrio* sp. (P11) took advantage of the host thalli using its motile ability hence can immediately attach and conquer seaweed tissue as an initial step of infection. Its ability to utilize carrageenan in the seaweed thalli as a carbon source by penetrating infected branches' medullary layer was another advantage that can cause ice-ice disease under stressful conditions.

Similarly, the ability of *Pseudoalteromonas carrageenovora* to produce kappa-carrageenase enzyme and degrade kappa-carrageenan in *K. alvarezii* indicated that this bacteria could trigger ice-ice disease symptoms like whitening of the thalli (Riyaz et al., 2020).

Table 1. Associated and pathogenic bacteria isolated from ice-ice infected seaweeds

Seaweed	Associated bacteria	Pathogenic bacteria (ice-ice disease promoters through pathogenicity test)	Method to confirm as ice-ice disease-causing bacteria	Location	References
Kappaphycus alvarezii and *Eucheuma denticulatum*	*Cytophaga-Flavobacterium* complex, *Vibrio* group	*Vibrio* sp. (P11), *Cytophaga* sp. (P25)	Pathogenicity test	Philippines	(Largo et al., 1995b)
K. alvarezii	*Pseudomonas cepacia, Vibrio alginolyticus, Pseudomonas diminuta, Plesiomonas shigelloides, Flavobacterium meningosepticum*	*V. alginolyticus*	Pathogenicity test	Indonesia	(Aris, 2011)
K. alvarezii	*Shewanella haliotis, Stenotrophomonas maltophilia, V. alginolyticus, Arthrobacter nicotiannae, Ochrobactrum anthropic, Catenococcus thiocycli, Pseudomonas aeruginosa, Bacillus subtilis* subsp. *spizizenii*	*S. maltophilia, V. alginolyticus*	Pathogenicity test	South Sulawesi, Indonesia	(Achmad et al., 2016)
K. alvarezii	*Alteromonas macleodii, Bacillus oceanisediminis, Pseudomonas stutzeri, Pseudoalteromonas issachenkonii, B. hunanensis, B. megaterium, Alteromonas marina, Aurantimonas coralicida, Rhodococcus rhodochrous*	*Alteromonas macleodii, Pseudoalteromonas issachenkonii, Aurantimonas coralicida*	Pathogenicity test	Karimunjawa island, Indonesia	(Syafitri et al., 2017a)
K. alvarezii	*Vibrio* sp., *Halomonas* sp., *Alteromonas* sp., *Aestuariibacter* sp., *Primorskyibacter* sp., *Thalassospira* sp., *Pseudoalteromonas* sp.	*V. alginolyticus*	Agarolytic analysis	Semporna, Sabah, Malaysia	(Azizi et al., 2018)
K. alvarezii	*Vibrio, Bacillus*	Not determined	Not confirmed	Kottapatinam, Rameswaram, Thondi, India	(Riyaz et al., 2019)
E. denticulatum	Dominant species: *Rivularia, Marinagarivorans, Lewinella*	Not determined	Not confirmed	Vietnam	(Kopprio et al., 2021)
K. striatus	Dominant species: *Vibrio, Cobetia, Marinomonas, Pseudoalteromonas*	Not determined	Not confirmed	Vietnam	(Kopprio et al., 2021)

Marine-Derived Fungi as Potential Causative Agents

Aside from marine bacteria, marine-derived fungi also play an essential role as ice-ice inducers. The only study that proved that marine fungi are also involved in this disease was Solis et al. (2010). They isolated 18 morphospecies of marine-derived fungi from *K. alvarezii* and *K. striatus* gathered from Calatagan, Batangas, Philippines. Ten marine-derived fungi were used in the ice-ice disease induction assay. The study showed that three isolates (*Aspergillus terreus, A. ochraceus,* and *Phoma* sp.) triggered ice-ice disease in healthy, non-axenic *K. alvarezii* cultures.

Control Measures

Proper Farm Management and Intervention

Malpractice of seaweed farming harms seaweed production, leading to ice-ice disease development in the seaweed farms. Thus, proper farm management could potentially eliminate or, if not at least, minimize ice-ice disease occurrence. These are the following management intervention as suggested in early studies;

a) Avoid overcrowding seaweeds in farming. Overstocking seaweeds in the farm offers pathogenic bacteria susceptibility and hinders light penetration (Largo, 2002).

b) Stick within optimum conditions for seaweed requirements. Changes drastically in water temperature and salinity must be avoided (Largo, 2002).

c) During summer, especially El Niño seasons where light intensity is extreme, planted seaweeds are advised to transfer to a deeper location where irradiance is enough to prevent photoinhibition (Largo, 2002).

d) For Lian Bay, China, the seaweeds are suggested to be planted in deeper areas from August- October during La Niña years (Pang et al., 2015) related to salinity fluctuations. This recommendation may apply to locations with similar weather conditions.

e) Identify and choose the species or strains that are resistant to ice-ice disease. According to Hurtado et al. (2008), *K. striatus* var. *sacol* is more resistant to ice-ice disease and even to epiphyte infestation than *K. alvarezii* var. *tambalang*. Brown varieties of *Kappaphycus* showed more resistance against ice-ice disease induced by *Vibrio* sp. pathogens than green varieties due to their higher antimicrobial compounds (Irmawati & Sudirjo, 2017). Tissue-cultured *K. alvarezii* produced from the laboratory is also unsusceptible to ice-ice disease and better resistant towards extreme temperatures of 30 and 35 °C than farmed seaweeds (Azizi et al., 2018). Also, *E. denticulatum* is more resistant than *K. alvarezii* against epiphyte infestation and ice-ice disease (Tisera & Naguit, 2009; Pang et al., 2015; Ndobe et al., 2020). Hence, these species/strains can be used as stocks for seaweed cultivation, especially in ice-ice outbreak times.

f) In areas where the ice-ice disease outbreak is already determined, avoidance of planting susceptible species/strains during the ice-ice outbreak can be done and instead engage in other livelihood sources for the meantime.

Polyculture With Other Seaweeds

Some studies show that the polyculture of *Kappaphycus* and *Eucheuma* with other seaweeds exhibits potential control effects against ice-ice disease. These seaweeds are;

a) *Achantophota spiciformis. K. alvarezii* can be co-cultured with *A. spiciformis*- owing to its antimicrobial property, and this alga protects against the ice-ice disease with only 0.062% ice-ice incidence compared to 50% monoculture of *K. alvarezii* (Tokan et al., 2015).

b) *Laurencia majuscule*. Polyculture of *Kappaphycus* or *Eucheuma* with *L. majuscule* may be done since this species produce metabolites that can inhibit ice-ice disease-causing bacteria (Vairappan et al., 2010).

c) *Eucheuma denticulatum*. This species is readily available and economically farmed in many seaweed-producing countries. Pang et al. (2015) suggested that there was a reduction in ice-ice disease incidence and epiphytes *N. savatieri* in *Kappaphycus* species during July – August when co-cultured with *E. denticulatum* – a species with a more efficient defense mechanism against the diseases owing to its volatile halocarbons production when under intense irradiance and CO_2-lacking conditions (Mtolera et al., 1996).

Using Antimicrobial Substances Extracted From Plants

Few authors have investigated the effectiveness of extracts from some mangrove species against ice-ice disease-causing bacteria in laboratory experiments. For instance, extract from the *Avicennia marina* leaves using methanol solvent exhibited potential use as an antimicrobial against four ice-ice disease-causing bacteria, *S. maltophilia, S. haliotis, V. alginolyticus,* and *P. aeruginosa* (Rahman et al., 2020). Also, 500 ppm of flavonoids extracts obtained from the fruits of *Sonneratia alba*

showed an antibacterial property against *V. alginolyticus* – one of the opportunistic pathogens that trigger ice-ice development in *K. alvarezii* (Sulistijowati & Karim, 2020). Furthermore, the extracts from the leave of *S. alba* at a concentration of 2.5 – 10 mg ml^{-1} have effectively hampered the growth of nine ice-ice disease-causing bacteria such as *Alteromonas macleodii*, *Bacillus oceanisediminis*, *Pseudomonas stutzeri*, *Pseudoalteromonas issachenkonii*, *Bacillus hunanensis*, *Bacillus megaterium*, *Alteromonas marina*, *Aurantimonas coralicida*, and *Rhodococcus rhodochrous* (Syafitri et al., 2017b).

The use of common lantana (*Lantana camara*) extracts to obstruct ice-ice disease and increase the growth of *K. alvarezii* has been evaluated by Patadjai et al. (2019) in the field experiment. Based on their results, pre-soaking the seaweeds in a 500 ppm solution of common lantana extract for 30 minutes before planting resulted in the absence of ice-ice disease compared to untreated seaweeds. Hence, it is recommended to apply this strategy as ice-ice disease control.

All plant extracts tested by different authors with promising results in inhibiting the growth of ice-ice disease-causing bacteria as well as those tested effective in the field reducing/eliminating ice-ice disease occurrence. This suggests that plant extracts may be useful and feasible as a treatment for the disease through short immersion of the seaweeds to the extract solutions before out-planting to farms.

Nutrient Enrichment

Since seaweeds are dependent on available natural nutrients in the farm, it is not deniable that seaweeds absorb these nutrients for growth and development. Once reach harvestable size, the farmer harvests, dries, and then markets them. Because of extensive farming here, nutrients are also being harvested and not return to the natural environment, in contrast to when the seaweeds are naturally rotting alone. These nutrients may become limited, resulting in low growth and even may result in ice-ice disease.

Nutrient enrichment using both organic and inorganic fertilizers has been proven efficient in minimizing ice-ice disease occurrence. In particular, when seaweed *K. alvarezii* immersed to 10 ppm of sodium nitrate for 12 hrs before planting into the open sea for 45 days, not only growth and carrageenan yield was improved but also decreased the occurrence of ice-ice disease to 8.75% compared with untreated seaweed (97%) (Luhan et al., 2015). A similar study also revealed that *K. striatus* enriched with 8.82 g L^{-1} ammonium phosphate solution for about 5 seconds to 1 minute and left covered overnight using canvass before out-planting significantly reduced the occurrence of ice-ice disease of up to 42% compared to untreated (78%) during ice-ice disease season parallel to what the farmers practiced and observed in the study site (Tahiluddin, 2018). Acadian Marine Plant Extract Powder (AMPEP) as organic fertilizer enhanced growth and carrageenan and also not only efficient in reducing the epiphytes infestation (Borlongan et al., 2011; Hurtado & Critchley, 2013; Loureiro et al., 2017b; Ali et al., 2020) but also lessening the ice-ice disease occurrence (Hurtado & Critchley, 2013; Illud, 2020) in *Kappaphycus* cultivation. The mode of application for AMPEP in *Kappaphycus* varies, as demonstrated by studies. For example, to reduce epiphytic *Neosiphonia* infestation and increase growth rate, Borlongan et al. (2011) soaked the *K. alvarezii* seedling in 0.1 g L^{-1} AMPEP before out-planting. Illud (2020), on the other hand, used both 0.01 g L^{-1} and 8.82 g L^{-1} of AMPEP enriched by following the same method of Tahiluddin (2018). The action mode on the efficacy of AMPEP in ameliorating deleterious outcomes of disease outbreaks is thought to be via elicitation of seaweed's natural defense mechanism against pathogens (Hurtado & Critchley, 2013; Loureiro et al., 2017b). However, precautions must be taken into account as inorganic fertilizer such as ammonium phosphate at a concentration of 6–9 g L^{-1} significantly reduced the carrageenan yield and viscosity of *K. striatus* by 4.81% and 1.83 cPs, respectively (Robles, 2020).

Using Genetic Engineering

Nowadays, in improving the horticultural crops, genetic engineering approaches renders countless applications for abiotic (heat, drought, and salinity) and biotic (insect-pest and pathogenic organisms) stress tolerance by using several genes like in providing immunity against biotic stress (*Cry* genes, trypsin inhibitors, protease inhibitors, cystatin genes, chitinase, osmotin, glucanase, defensin, pathogenesis-related genes, and RNAi technique), and in giving protection against adverse environmental stresses (genes encoding for stress biosynthesis protecting compounds such as glycine, betaine, mannitol, and heat shock proteins, various transcription factors like mitogen-activated protein kinase (MAPK), WRKY, DREB1, etc.) (Parmar et al., 2017).

However, the application of genetic engineering in seaweeds for disease controls is still in its infancy. For instance, gene transformation to produce a seaweed resistant to abiotic stress as a potential solution for the ice-ice disease has been tested by Sulistiani et al. (2019) using the Gα gene extracted from soybean (*Glycine max* mar Slamet). Sulistiani et al. (2019) introduced this gene into the *K. alvarezii* callus using *Agrobacterium tumefaciens* and regenerated modified callus

cells to transgenic plantlets. Results revealed that *K. alvarezii* transgenic plantlets with Gα genes were successfully created. Likewise, Handayani et al. (2014) established a binary plasmid bearing chicken lysozyme gene and transferred it to *K. alvarezii* thalli using *A. tumefaciens* and also successfully produced a transgenic *K. alvarezii.* According to the authors, the Gα gene encodes for the heterotrimeric G protein α subunit is a gene that has an essential role in biotic and abiotic stress tolerance. Also, chicken lysozyme is a significant constituent of immune defense against pathogenic bacterial diseases that can boost seaweed tolerance against pathogens.

However, the authors of these two experiments did not evaluate the transgenic plantlets' efficacy in protecting against ice-ice disease. But their findings are beneficial for further investigation in the level of tolerance against ice-ice disease-causing bacteria and abiotic stresses.

Conclusion

The ice-ice disease is a disease caused by drastic fluctuations of water parameters in the environment, further degrade the stressed branches by the attack of pathogenic microorganisms. The occurrence of this disease varies from species, places, and seasons (wet and dry). Control measures may include proper farm management, polyculture with other seaweeds, pre-soaking with antibacterial substances, nutrient enrichment, and possibly using genetic engineering. Knowledge of this disease may be helpful, especially for the farmers, to avoid production losses and improve the seaweed industry.

Compliance with Ethical Standards

Authors' Contributions

ABT and ET conceptualized the study, ABT wrote the first draft of the manuscript and then ET edited the manuscript. Both authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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RESEARCH ARTICLE

Time-dependent change of the digestive enzyme activity of Black Sea salmon (*Salmo labrax* Pallas, 1814) fed at suboptimal temperature

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ABSTRACT

The present study conducted at 10.21±0.27°C water temperature in freshwater recirculating aquaculture systems (RAS) to observe the temporal variations in the digestive enzyme activities including pepsin, trypsin, amylase and lipase of Black Sea salmon (*Salmo labrax*). Seventh filial generation (F7) of Black Sea salmon (*Salmo labrax*) with average initial weights of 69.85±10.08 g were by hand fed three times daily until apparent satiation. At the end of the 75-day trial the samples were dissected that reached a weight of 179.17±31.08 g at 45th minute, 3rd, 6th, 12th, 24th, 36th, 48th, 72nd and 96nd hours post feeding. In all enzyme groups, the third hour after feeding was recorded as the time when the highest levels were observed. However, enzyme activities decreased gradually as the time after feeding was prolonged. In the nutrition studies to be conducted at a suboptimal temperature in RAS, gut sampling of Black Sea salmon can be taken at 3rd hour after feeding. For a better understanding of digestive enzyme activity for this species, however, different sections of the digestive system should be comprehensively monitored including different temperature conditions.

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Introduction

The digestive system of fish covers the region from mouth to anus. This anatomical structure is usually divided into headgut, foregut, midgut and hindgut (Floris, 2010). Chemical digestion of feeds starts in the foregut and continues in the midgut, even the midgut is where absorption takes place (Banan Khojasteh, 2012). Fish increase feed efficiency by digesting nutrients in the feed with the help of digestive enzymes (Shabana et al., 2019). The degradation of nutrients in the alimentary canal of fish is mostly dependent on the enzymes present (Hani et al., 2018). Digestive enzymes have a vital role

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in the digestion of proteins, lipids, and carbohydrates. The absorption of digested substances from the intestinal wall for the growth and reproduction of the fish is facilitated by digestive enzymes (Amhamed et al., 2018). Most fish have seven major digestive enzymes like trypsin, carboxypeptidase a, carboxypeptidase b, maltase, amylase, lipase, and alkaline phosphatases (Hani et al., 2018). Among these enzyme groups, amylase, lipase and trypsin enzymes are the three main digestive enzymes that operate in the intestines to regulate digestion in animals. Therefore, the activities of digestive enzymes in the intestine represent the digestive status of the fish (Xu et al., 2019). The digestive enzyme activities of fish are influenced by various factors such as diet and feeding habits, fish age, growth stage, pH, and temperature, fish species, and digestive system structure (Amhamed et al., 2018). Among these factors, the temperature is one of the most important critical factors that directly affect the reproduction, survival, growth, nutrient intake, nutrient efficiency, and oxygen consumption of fish (Sun et al., 2015). Since the effectiveness of digestive enzymes highly reflects changes in diet, knowing the nutritional habits of different fish species associated with digestive enzyme activities in the gastrointestinal system is important to provide an appropriate diet for each species (Gioda et al., 2017). Therefore, determining digestive enzyme activity contributes to understanding the digestive physiology of fish (Hani et al., 2018).

Black Sea salmon, *Salmo labrax*, is a subspecies of the brown trout distributed at the Eastern Black Sea, and an opportunistic ecotype. Black Sea salmon is an endemic species for Turkey. This species of is represented by three different ecotypes: (i) sea ecotype: that migrates to the sea in the Black Sea basin, (ii) stream ecotype: that does not migrate to the sea but resident in the stream in the Black Sea Region, and (iii) lake ecotype: that residents in lakes in the Black Sea Region (Tabak et al., 2002). Under Black Sea conditions, following stripping, Black Sea salmon are grown in freshwater ponds until smolt stage (about 12 cm and 15 g). Individuals who reach the smolt are smoltified by transferring to seawater. Fish are kept during the periods (November-May) allowed by the water temperature in marine cages. In May, when the sea water reaches its critical value (18°C) of the temperature for Black Sea salmon aquaculture, the fish are harvested or transferred to freshwater ponds again for broodstock maintenance. Additionally, in the dam lakes where the water temperature, water depth and water current are suitable for aquaculture, Black Sea salmon can also be produced throughout the year or at a certain period of the year (SUMAE, 2010). The juvenile Black Sea salmon, which are grown up to 2-5 g in freshwater ponds, can be grown up to 350-400 g before the fillet size by transporting to the dam lake cages. During the months when the sea water temperature is suitable for Black Sea salmon production, fish can be reached fillet size by transporting from the dam lake cages to the marine cages. Moreover, Black Sea salmon can also be grown in freshwater ponds throughout all year without being transported to marine and dam lake cages. During these production processese, optimal and non-optimal water temperatures in both freshwater and sea water occur depending on the season. This condition can lead to the creation of different maintenance and feeding programs in aquaculture. Therefore, it is important to know the changes that occurred in the digestive physiology and metabolism of the species at optimum or non-optimum temperatures. This study aimed to determine the effect of suboptimal temperature on digestive enzyme activity in the gastrointestinal tract of Black Sea salmon.

Material and Methods

Material

The present study conducted between November 2018 and February 2019 at freshwater recirculating aquaculture systems (RAS) at Central Fisheries Research Institute, Trabzon, Turkey. Seventh filial generation (F7) of Black Sea salmon *Salmo labrax* (Pallas, 1814) with average initial weights of 69.85±10.08 g were selected for the study. In November 2019, fish were placed randomly in 500 l tanks. The trial was performed as triplicates containing 110 fish per tank. The fish were hand fed three times a day at 08:00, 12:00 and 16:00 for 75 days until apparent satiation. The basal diet used in the study was shown in Table 1. Water temperature (10.21±0.27°C), pH (7.09±0.28) and oxygen (9.39±0.32 mg/l) were recorded three times a day. Ammonia (0.03±0.02 mg/l) was measured weekly. Water changes in the tanks were conducted 20 times a day. Cleaning of tanks was performed by siphoning daily. The sampling was carried out according to both the European Union Directive (2010/63/EU) (European Commission, 2010) and ARRIVE ethical guidelines (Kilkenny et al., 2010). The study was performed with the approval of the Ethical Committee of Animal Experiments of Central Fisheries Research Institute (coded as ETIK-2017/1).

Methods

The tissue sampling was carried out at 08:00. For this, the midgut part of the intestine tissues from the fish was dissected at 45th minute (min.), 3rd, 6th, 12th, 24th, 36th, 48th, 72nd and 96nd hours (h) post feeding. Tissue samples were stored at -80°C until analyzed. Then, they were brought to Çanakkale Onsekiz Mart University, Faculty of Arts and Science, Biology Department, Water Ecology Laboratory in the cold chain. It

was necessary to prepare homogenate from the tissues to be used and to obtain cytosolic fractions to analyze the digestive enzymes. The tissues taken were weighed and homogenized in a 1:5 ratio with homogenization buffer (0.05 phosphate buffer pH 7.4). The specific activity of each enzyme evaluated in the study was measured spectrophotometrically. Bradford (1976) method was used to calculate the amount of protein.

Table 1. Formulation and proximate composition of the experimental diet

Ingredients	**%**
Fish meal	31
Soybean meal	20
Wheat gluten	6
Pea protein	12
Sunflower seed meal	7
Wheat flour	12.5
Fish oil	11
Vitamin mix[1]	0.22
Mineral mix[2]	0.16
Vit C	0.12
Proximate Composition	
Crude protein	46.31
Crude lipid	14.91
Crude ash	9.34
Moisture	6.19

Note: [1]Supplied the following: inositol 300 mg, biotin (Vit B7) 200 mg, tocopherol (Vit E) 200 mg, calcium pantothenate (Vit B5) 50 mg, riboflavin (Vit B2) 30 mg, pyridoxine (Vit B6) 20 mg, thiamine (Vit B1) 20 mg, menadione (Vit K3) 12 mg, niacin (Vit B3) 6 mg, retinol (Vit A) 0.6 mg, folic acid (Vit B9) 0.5 mg, cholecalciferol (Vit D3) 0.05 mg, cobalamin (Vit B12) 0.05 mg. [2]Supplied the following: ferric sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) 50 mg, manganese (II) oxide (MnO) 50 mg, zinc oxide (ZnO) 50 mg, copper sulfate pentahydrate ($CuO_4S \cdot 5H_2O$) 10 mg, calcium iodate (Ca_2IO_6) 0.8 mg, cobalt carbonate hexahydrate ($CoCO_3 \cdot 6H_2O$) 0.15 mg, sodium selenite (Na_2SeO_3) 0.15 mg.

Trypsin enzyme activity: The measurement was done due to Tseng et al. (1982) analysis method and Na-Benzoyl-DL-arginine-p-nitroanilide (BAPNA) was used as substrate. Enzyme activities of the samples were measured in a spectrophotometer at 253 nm wavelength for 5 minutes.

Pepsin enzyme activity: The measurement was performed using a revised version of the analysis method used by Worthington (1982) by Infante & Cahu (1994). Besides, bovine hemoglobin was used as a substrate. Samples were measured at a wavelength of 280 nm for 5 minutes.

Amylase enzyme activity: The enzyme levels were obtained depending on the study conducted by Bieth & Metais (1968) they used soluble starch as a substrate. Samples were measured at 540 nm wavelength for 5 minutes.

Lipase enzyme activity: α -naphthyl caprylate was used as the substrate, and the analysis method was used in the study conducted by Versaw et al. (1989). The measurements were done at 490 nm wavelength for 10 minutes.

Statistical Analyses

The descriptive statistics were presented as mean±Sx. Data were statistically analyzed by the one-way analysis of variance (ANOVA) procedure of SPSS 21.0 (Table 1). Duncan's multiple range test was performed for the significance of differences of means between groups. Probability levels of $p<0.05$ were chosen for statistical significance.

Results

Table 2 presents the results obtained from the digestion enzyme activities at suboptimal temperature. Significant differences were observed in the digestive enzyme activities of Black Sea salmon (One way ANOVA, $p<0.001$). The highest enzyme activity was obtained in pepsin (144.57±9.64 U mg^{-1}). This was followed by trypsin (60.64±11.24 U mg^{-1}) and amylase (3.69±0.42 U mg^{-1}). The lowest enzyme activity was obtained in lipase (0.04±0.00 U mg^{-1}). According to the results, the highest activity for all enzymes was observed at 3rd-hour post feeding. Digestive enzyme activities increased rapidly until 3 hours post feeding. After 3rd-hour post feeding, all enzyme activities tended to decrease over time. The decrease in amylase enzyme was faster than other enzymes. But, the increase in amylase enzyme was faster than other enzymes until 3rd-hour post feeding (Figure 1). From the 3rd hour to the 96th hour, the activity of pepsin, trypsin, amylase and lipase enzymes decreased in 95.45, 93.50, 95.12, and 95 % levels, respectively. In addition to, from the 45th minute to the 3rd hour, the activity of pepsin, trypsin, amylase and lipase enzymes increased in 56.07%, 64.10%, 78.86%, and 50% level, respectively.

When the correlations between digestive enzyme activities were examined, it was determined that the correlation between all measured digestive enzymes in specimens was found to be significant (Table 3), and the correlations between these enzymes were found to be strong in the positive direction.

Discussion

The activity of digestive enzymes in fish species can be influenced by factors including fish age, type of feeding, temperature (Munilla-Moran & Saborido-Rey, 1996), fish size, season, and origin (Hani et al., 2018). Temperature is one of the

Table 2. Time-dependent change of digestive enzyme activities of *S. labrax*, U mg^{-1}

Time	Pepsin	Trypsin	Amylase	Lipase
45min.	50.32±3.40^{c}	21.77±0.95^{b}	0.78±0.12^{b}	0.02±0.00^{b}
3h.	114.57±9.54^{a}	60.64±11.24^{a}	3.69±0.42^{a}	0.04±0.00^{a}
6h.	82.42±3.77^{b}	26.40±1.56^{b}	0.66±0.04^{bc}	0.02±0.00^{b}
12h.	45.96±2.93^{c}	21.32±1.61^{b}	0.35±0.01^{cd}	0.02±0.00^{b}
24h.	28.17±3.03^{d}	17.73±1.29^{bc}	0.24±0.01^{d}	0.01±0.00^{c}
36h.	19.54±3.05^{de}	9.96±1.07^{cd}	0.21±0.01^{d}	0.01±0.00^{c}
72h.	15.50±2.02^{ef}	6.45±0.82^{d}	0.18±0.01^{d}	0.01±0.00^{d}
96h.	5.29±0.88^{f}	3.94±0.46^{d}	0.18±0.01^{d}	0.002±0.00^{d}
P values	0.000	0.000	0.000	0.000

Note: Mean values in row with different superscripts were significantly different (P<0.001). Values are given as mean±Sx (n=5).

Table 3. Pearson correlation coefficients between the specific activity of digestive enzymes of the *S. labrax* (n=38)

Enzymes	Pepsin	Trypsin	Amylase	Lipase
Pepsin		0.84**	0.76**	0.92**
Trypsin	0.84**		0.84**	0.81**
Amylase	0.76**	0.84**		0.79**
Lipase	0.92**	0.81**	0.79**	

Note: **Correlation is significant at the 0.01 level (2-tailed).

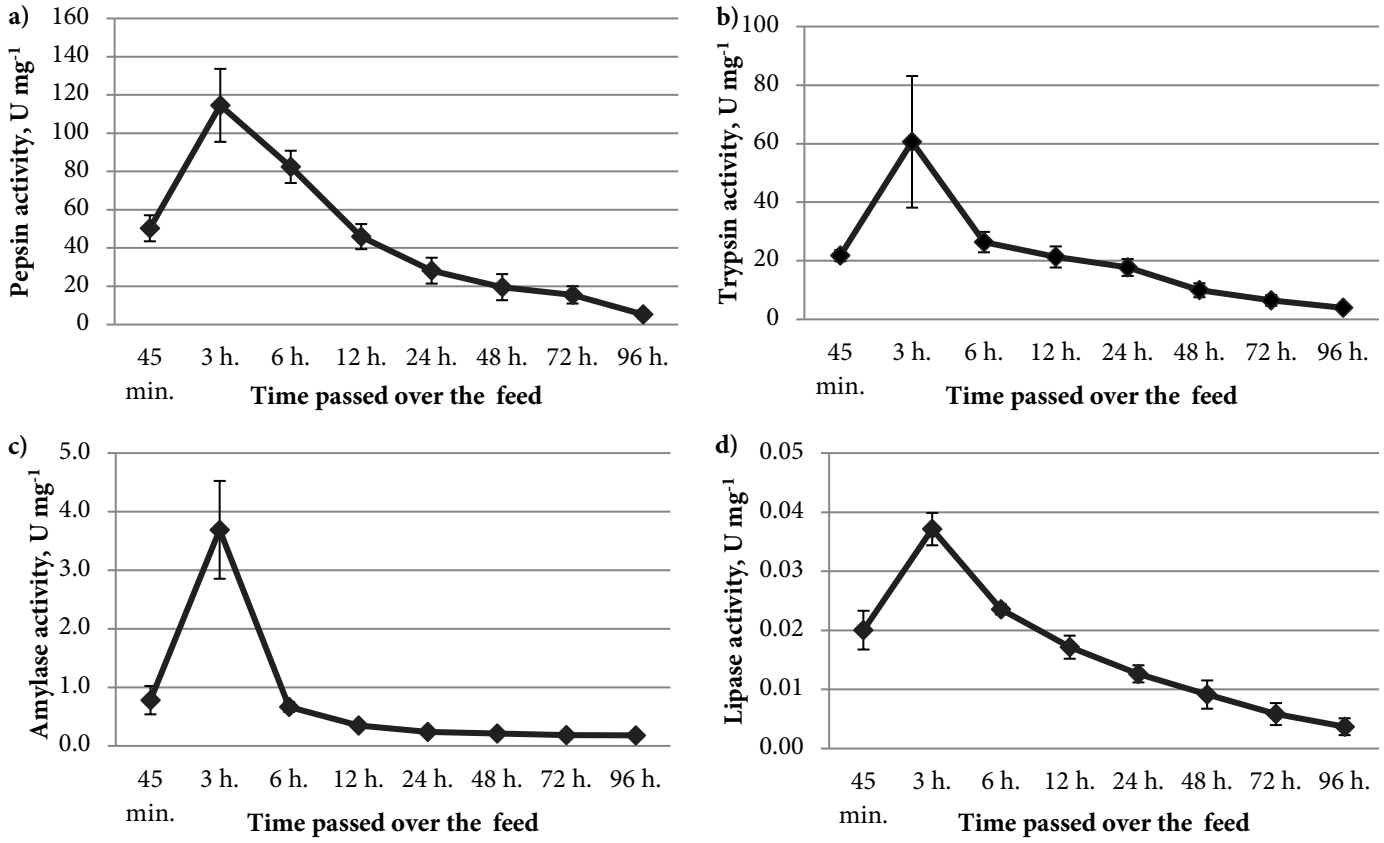

Figure 1. Time-dependent change of digestive enzymes post feeding, a: Pepsin, b: Trypsin, c: Amylase, d: Lipase.

most important external factors affecting the metabolic rate in ectotherm animals and has a direct effect on fish activity. At suboptimal temperature, the activities of various enzymes may decrease, which may result in decreased growth rate, and reduced digestibility of feeds (Bowyer et al., 2014). In our study, pepsin, trypsin, amylase, and lipase activity of Black Sea salmon were significantly influenced by time passed post feeding. A similar result was seen in Özel & Gürkan (2019). In addition, Gheisvandi et al. (2014) indicated that the activities of trypsin and amylase of Caspian kutum (*Rutilus kutum*) larvae fed at

18.4°C were highest at 8-hour post feeding. Our study demonstrated that trypsin, pepsin, amylase, and lipase enzymes increased from 45th minute to 3rd-hour post feeding, but decreased after 3rd hour. Unlike our study, Özel & Ertürk Gürkan (2019) found that the highest digestive enzyme activities in Black sea trout fed at 15°C were in 45th-minute post feeding. Hani et al. (2018) stated that most aquatic organisms have the ability to adapt to different temperatures because they are ectothermic animals. Miegel et al. (2010) stated that water temperature can have a direct effect on feed intake and enzyme activity. Intestine motility decreases at low water temperatures. This condition can result in higher intestinal enzyme activity (Miegel et al., 2010). Ahmad et al. (2014) found that the lipase and trypsin activities of *Clarias batrachus* were found to be the lowest at 10°C, which is the lowest temperature. Unlike the Özel & Ertürk Gürkan (2019), pepsin enzyme activity in the digestive system was higher at suboptimal temperature in our study, but amylase was lower. Kofuji et al. (2005) found that pepsin activity in the stomach of yellowtail (*Seriola quinqueradiata*) was lower in the colder water temperatures, but trypsin activity in the intestine was higher. The difference from our result could possibly be due to the sampling section of the gastrointestinal tract, water temperature, and also fish species. Gabriel et al. (2017) stated that the distribution and activities of digestive enzymes such as pepsin, trypsin, amylase, and lipase are at different levels throughout the gastrointestinal tract. This depends generally on the nature and composition of the diet. Regarding these studies, we can also say that the effect of suboptimal temperature on the activity of digestive enzymes may be at different levels depending largely on the digestive physiology of the fish species. Indeed, Wei et al. (2010) stated that the activity of digestive enzymes is an important indicator in understanding the digestive physiology of fish species.

Conclusion

We found that activity of digestive enzymes (pepsin, trypsin, amylase, and lipase) in the midgut section of the intestine of Black Sea salmon fed at 10.21°C in culture condition reached the highest level by increasing 3rd-hour post feeding. Enzyme activities decreased gradually by time after the 3rd hour. Among digestive enzymes, the highest activity was seen in the pepsin enzyme. This was respectively followed by trypsin and amylase. The lowest activity was seen in the lipase enzyme. We suggest that to determine digestive enzyme activities in nutrition researches to be conducted at the suboptimal temperature, the midgut intestine tissues of Black Sea salmon (appr. 179.17 g) can be taken at 3rd-hour post feeding. However, in order to better understand the effect of suboptimal temperature on the digestive enzymes of the species in the gastrointestinal tract, it is necessary to study the stomach, pyloric caeca and anterior, middle and posterior intestines should be monitored separately by supported with nutrition studies.

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Compliance With Ethical Standards

Authors' Contributions

Author OTÖ did conceptualization, methodology, design of the experiments and feed formulations, experiments, feeding studies and manuscript writing, SEG determined digestion enzyme activity, OTÖ and SEG performed data analysis, validation, and review.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

The authors confirm that the ethical policies of the journal have been adhered to and the appropriate ethical review committee approval has been received from the Animal Ethics Committee of Central Fisheries Research Institute, Turkey (application number ETIK-2017/1). The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes and feed legislation.

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RESEARCH ARTICLE

The effect of different processing methods on fishmeal element quality: Evaporator system

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ABSTRACT

Anchovy (*Engraulis encrasicolus* L., 1758) is the main source of fishmeal in Turkey. The research was carried out in 3 fishmeal factories where anchovy is processed in the Black Sea during the 2007-2008 fishing season. Factories A and B have an evaporator system, while factory C does not have an evaporator system. In the study, it was aimed to reveal the effect of the evaporator system on the mineral substance quality of the fishmeal produced in fishmeal oil factories. As a result of this study, the elements in fishmeal are listed in descending order as Ca>P>K>Na>Mg>Fe>Zn>Mn>Cu. According to the results of the research, it was determined that the phosphorus, sodium, potassium, calcium and zinc values of fish fishmeal produced in A and B factories using evaporator system were higher than the C factory without an evaporator system, and the difference between factories was statistically significant ($P<0.05$). It is recommended that all factories have an evaporator system in order to produce fishmeal of higher quality (protein and mineral substances) in fishmeal-oil factories.

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Introduction

The consumption rate of seafood, which is very significant in the healthy food of people, is constantly increasing (Bayrakli & Duyar, 2021). Fish is one such source that provides 17% of animal protein to the world's population (Halden et al., 2014). Given the forecasted 9 billion in world population by the year 2050, fish becomes an indispensable reliable source of nutrients as aquaculture is also fast-growing (Halden et al., 2014; FAO 2014). In this increase, the contribution of aquaculture is too much to underestimate (Tidwell & Allan, 2001; Duyar & Bayraklı, 2005).

In the world, approximately 96.5 million tons/year of aquaculture is obtained by fishing (FAO, 2020). While it is assumed that there will be a reduction due to both overfishing

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and pollution in the coming years; it is thought that the nutritional needs of people will be met by aquaculture obtained through aquaculture. The aquaculture sector has caught the amount of fishing today by catching a rapid rise after the fish and shrimp producers used fishmeal and oil in their feed mixes in the 1980s (Ruiter, 1995; Bayraklı & Duyar, 2019a). The increase in aquaculture also increases the demand for fishmeal and oil (New & Wijkströem, 2002).

The composition of commercial feeds used in feeding cultured fish also affects the mineral composition of the fish. Minerals represent a minor proportion in the feeds' composition. However, they are required in almost every aspect of animal metabolism. Minerals are classified as macro elements that the body needs in large quantities or microelements that the body needs in small quantities (FAO, 2017). Macro elements include phosphorus, calcium, potassium, magnesium, sodium, chlorine, and sulfur, while microelements are composed of copper, zinc, manganese, iron, cobalt, and iodine, among others (Antony et al., 2016; FAO, 2017).

Minerals such as calcium, phosphorus, magnesium, sodium, potassium, sulfur, chlorine, iron, copper, cobalt, iodine, manganese, zinc, molybdenum, selenium, and fluorine play a role in biological functions as elements or compounds (Makwinja & Geremew, 2020). If these mineral substances are not taken with the feed, they may be the cause of development and disease in the fish that are fed (Bilal & Dryer, 2021).

Minerals in food are present depending on salt and proteins (such as phosphorus in phosphoproteins and metal ions in enzymes). Minerals in foods are very important not only in terms of nutritional physiology but also in terms of taste (Telefoncu, 1993). Mineral composition in marine organisms varies according to seasonal and biological differences (species, height, black and white muscle, sex, sexual maturity), fishing area, processing method, food source, environmental impact (water chemistry, salinity, temperature, and contamination) (Rodrigo et al., 1998; Alasalvar et al., 2002). Fish meats are a valuable source of calcium and phosphorus, as well as zinc, iodine, potassium, iron, copper, magnesium, and selenium (Pigott & Tucker, 1990; Sikorski et al., 1990).

Each year, about 20 million tons of raw materials come from whole fish, shellfish, wild fish by-products, and farmed fish by-products. These raw resources can produce about 5 million tons of fishmeal, about 1 million tons of fish oil (Bayraklı & Duyar, 2019b).

Almost all fishmeal is made by cooking, pressing, drying, and grinding the fish in machinery designed for the purpose. Although the process is simple in principle, considerable skill and experience are necessary to obtain a high yield of high-quality products and to make the plant efficient. A typical process is shown diagrammatically in Figure 1.

In fishmeal oil factories, first separation and then evaporation processes are carried out in order to separate the aqueous, oily, and solid parts from each other after the pressing process (Figure 1). The evaporator required for this process may not be integrated into factories or sometimes not operated due to its high cost and high operating fee (Bayraklı & Duyar, 2021). It is thought that these different applications can change the element structure of fishmeal.

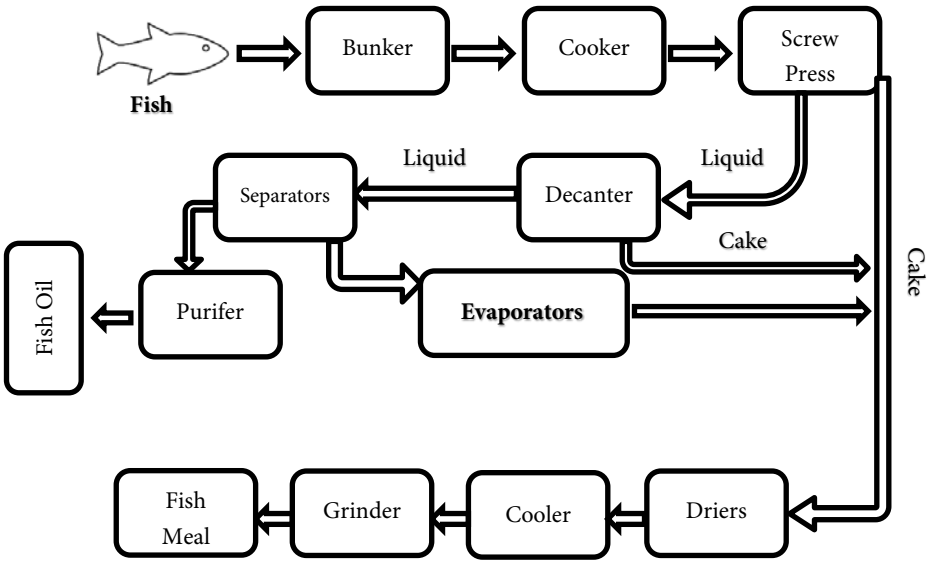

Figure 1. Process diagram of the fish meal-oil production line

Table 1. Minerals detected in fishmeal and some literature (mg kg^{-1})

		Fishmeal	Ca	Na	K	P	Mg	Fe	Zn	Mn	Cu
In research	A	AM	7114	10105	29458	26644	264.83	2525	247.62	16.78	2.20
	B	AM	6391	10940	29360	24608	324.12	2146	253.04	20.12	2.21
	C	AM	4578	8087	26774	23976	291.68	2032	232.76	18.35	2.21
Satoh et al. (1987)		SA	44300	3200	3500	27300	2000	239	141	11.1	5.4
		Fb-PM	52200	8400	3100	29300	2800	97.3	71	5.2	11.5
		BHM	34400	8300	13400	24700	2000	289	141	11.1	5.4
Moghaddam et al. (2007)		SM	39700	8300	5200	26100	2700	229	74.5	3.7	6.2
		HM	22900	6100	10900	17000	1500	140	132	5	6
		AM	37300	6500	6900	24300	2400	220	103	10	9
		MFM	51100	6500	6500	28800	1600	440	147	33	11
Storebakken et al. (2000)		HM	23900	14500	12200	22100	2640	155	121	5	3.26
Cho et al. (1987)		AM	6772	20630	7143		2222	3598	167		6
		KFM	10900	8801	7453		2954	4092	190		163
Zarkadas et al. (1986)		HM	19500	4200	12000	15000	1100	100	100	30	
Irungu et al. (2018)		FWSM	3932.4	21246.1	10065.2	4385.8	2191.5	922.2	454.9	1932.1	1512.5
		ACM	1600.7	22633.6	6542.5	6542.5	1467.7	931.9	566.4	3126.1	1628.8

Note: AM: Anchovy meal, Fb-PM: Fish by-products meal, BHM: Brown fish meal, SA: Sardine meal, SM: Shrimp meal, HM: Herring meal, MFM: Menhaden fish meal, KFM: Kilka fish meal, HFM: Hake fish meal, FWSM: Freshwater shrimp meal, ACM: Adult cricket meal.

It is very important to know the element structure of fishmeal, which is the raw material of fish feed, which is essential especially in aquaculture. In this study, the effect of the evaporator system found in some fishmeal-oil factories on the mineral amount of fishmeal produced was investigated.

Material and Methods

Material

The research was carried out in 3 fishmeal factories that process anchovy (*Engraulis encrasicolus L.*), which were caught in the Black Sea during the 2007-2008 fishing season. Factories A and B have an evaporator system, while factory C does not have an evaporator system. Factories operate in the same area. Since anchovy is a type of fish that feeds, wintering, and breeding in the north-south and east-west directions in the Black Sea (Özdemir et al., 2010), the fish used in the research are also the material that is caught from the same region.10 pieces of 1 kg fishmeal samples were taken from each factory on the same day and delivered to the laboratory for analysis.

Analytical Technique

For mineral substance analysis, homogenized fishmeal samples were weighed 0.1 g in high pressure resistant teflon containers (10ml volume, 200 psi pressure-resistant), then 4ml nitric acid (65%) (Merck, 1.00452) was added to them. Wet combustion was applied to Teflon containers in a closed system microwave oven. In the first 15 minutes, a temperature of 150°C was reached gradually, and it was kept at this temperature for 5 minutes. After the pressure dropped below 50 psi, the temperature was allowed to reach 250°C within 15 minutes and it was kept for 5 minutes under these conditions. After waiting for cooling at the end of wet burning, the burned samples were washed with ultrapure water, taken into 14 ml falcon tubes, and completed with ultrapure water. Then, readings were made on the Perkin Elmer ICP-OES Optima 2100 DV spectrometer as stated in EPA (1994).

Experimental Plan and Statistical Analysis

A wholly randomized experimental design was used. Data were exposed to analysis of one way variance and Duncan's multiple range tests using the SPSS version 15 statistical package adapted to a personal computer.

Results and Discussion

The composition of commercial feeds used in feeding the cultured fish affects the mineral composition of the fish (Çaklı, 2007). In this study, the element content of fishmeal obtained from 3 factories was determined

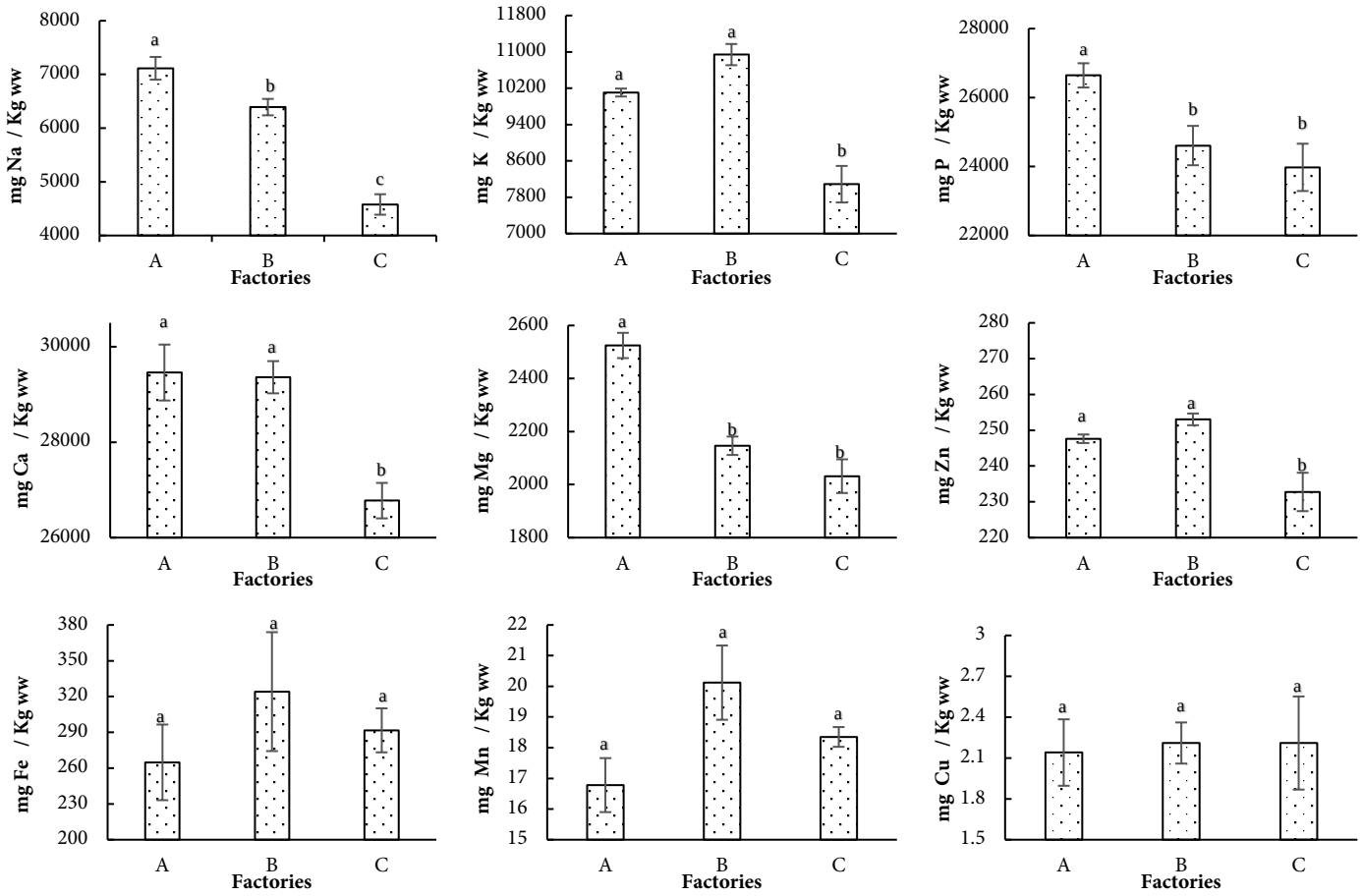

Figure 2. Mineral maters were detected in A, B, and C fishmeal factories (Different lowercase letters (a - c) in succession indicate significant differences ($p<0.05$) between A, B, and C factories that process fishmeal by different methods)

(Ca>P>K>Na>Mg>Fe>Zn>Mn>Cu). The findings of the researchers who reported the mineral substance values obtained in the study and the mineral substance amounts in fishmeal obtained from different raw materials are shown in Table 1. All researchers reported that there are the most calcium and phosphorus minerals in fishmeal, followed by sodium, potassium, and magnesium minerals. These results are similar to our study. The reason why different researchers find the same mineral values different is because of the seasonal and biological differences (species, height, dark and white muscle,sex, sexual maturity, breeding time, water chemistry, salinity, temperature) in marine organisms, the method of processing, fish (Bayrakli, 2021). It can be said that the fishmeal varies depending on whether it is made from whole fish or from processing waste.

As seen in Figure 2; from the mineral matter analysis results of fish meal produced in A, B and C factories for P (26644.25±351, 24608.38±570, 23976.38±686 mg kg^{-1} ww), Na (7114.42±211, 6391.44±153, 4578.31±190 mg kg^{-1}), K (10104.75±870, 10939.75±233, 8086.88±402 mg kg^{-1} ww), Ca (27256.36±586, 29360.65±337, 26774.74±372 mg kg^{-1} ww), Zn (247.62±120, 253.04±1634 and 232.76±536 mg kg^{-1} ww) were found, respectively. As a result of the mineral matter analyzes performed on fish meal produced in A, B, and C factories, Ca, P, K, Na, and Zn elements in A and B factories with an evaporator system were found to be significantly higher than the C factory. The difference between factories with and without evaporator systems was found to be statistically significant ($p<0.05$). Cu element was found similar in all three factories, statistical difference between factories was found to be insignificant.

The statistical difference between the factory groups of the average Fe, Mn, Cu elements (average 293.55±20.343, 18.42±0.565, 2.18±0.142 mg kg^{-1} ww, respectively) in fishmeal produced in A, B, and C factories was found to be insignificant ($p>0.05$).

Especially in A and B plants using evaporators, phosphorus, sodium, potassium, calcium, and zinc were found to be of high quality compared to C factory, which does not use an evaporator system, as much as the difference between the couple is statistically significant ($p<0.05$). While the cake piece goes directly to the dryer as a result of the press operation from the fishmeal processing stage, the liquid cake part goes to the evaporator and the solid release is included after the

evaporation. Due to this process, more information on sodium, potassium, calcium, and zinc is obtained in factories with evaporators. In the factory that does not use an evaporator system, these minerals are left as waste after oil is taken. P, Na, K, Ca, and Zn elements that dissolve in water and have nutritious properties remain on the cake as a result of the evaporation of the water in the evaporator and are added to the fishmeal. In this way, the evaporator system contributes 3.5-4% to the total fishmeal (Duyar & Bayraklı, 2005).

Conclusion

Fishmeal is also a valuable source of the minerals sodium, calcium, phosphorous, magnesium, potassium and of trace elements, notably zinc, iron, copper and manganese. The significance of trace minerals as essential ingredients in diets, although in small quantities, is also evident in fish. Minerals are required for usual life processes, and all animals, with fish, need these elements. Fish may derive these minerals from the diet and also from ambient water. The minerals are accountable for the skeletal formation, conservation of colloidal systems, regulation of acid-base equilibrium, and biologically important compounds such as hormones and enzymes. Mineral deficiencies can cause biochemical, structural, and functional pathologies which depend on some factors, including the duration and degree of mineral lack (Watanabe et al., 1997).

Marine resources used to produce fishmeal and fish oil are finite resources that have been fully utilized for decades. Aside from higher recovery and utilization of seafood processing byproducts, there is no prospect of increasing fish meal and fish oil production from wild stocks of marine fish (Naylor et al., 2009). Fish meal is a complex material containing a wide array of essential nutrients and biologically active compounds, many of which are absent in plant proteins.

It is getting harder and harder to reach raw materials every day. There is an obligation to process our raw materials in a more efficient and beneficial way for consumers by using constantly developing technologies (Bayraklı & Duyar, 2019b). Phosphorus, potassium, calcium, sodium, and zinc minerals detected in fishmeal were found to be high in factories operating with evaporator systems. It has been determined that the evaporator system increases both the amount and quality of the final product obtained from the raw material for fish meal and oil factories. For these reasons, it is recommended that all fish meal oil factories have and use an evaporator system.

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Compliance With Ethical Standards

Authors' Contributions

Author HAD designed the study and wrote the first draft of the manuscript, BB performed and managed statistical analyses. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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RESEARCH ARTICLE

Competitive power of Turkey's aquaculture sector and comparison with other leading countries

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ABSTRACT

In the study, the production, foreign trade, and competitiveness of the countries, which have an important share in world aquaculture production, were examined. According to the 2019 data shows that Chine and Indonesia are the most important aquaculture producers in the world. The data set belonging to 2010-2019 was used in the research. In the study, Revealed Comparative Advantage (RCA), Vollrath Relative Export Advantage Index (RXA), Relative Trade Advantage Index (RTA), Relative Competitiveness Index (RC), Relative Import Advantage Index (RMA), and Trade Balance Index (TBI) indices were used. According to the index results, it is concluded that Turkey is advantaged in terms of fresh and chilled fish foreign trade competition. Turkey has an importer position in world frozen fish and other aquaculture trade.

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Introduction

Archeological remains show that products obtained as a result of fishing and pisciculture have been used as a staple food due to the vital activities of humanity since the early ages (OKA, 2013; Candemir & Dağtekin, 2020). Today, the need for food with the increasing world population has led to important developments in the fishing industry (Anticamara et al., 2011). Thanks to these developments, the aquaculture sector has employed millions of people as a result of economic activities such as production, processing, conservation, and transportation. The upward trend in the production and trade of aquaculture products across the world has enabled an increase in the number of people employed in this sector (Bashimov & Aydın, 2018). Today, the aquaculture sector -with the employment and income revenue volume that it has created- is one of the sectors with great economic value (Turan et al., 2006; Tatlıdil et al., 2009; Saygı et al., 2015).

When the countries with the greatest foreign trade volume are examined, China which is the greatest producer of aquaculture products is also observed to hold the leading position in the exportation of them. Again, it is interesting that European countries and Canada, which are not among the important producers, rank among the first 10 exporters. While

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the majority of these countries are at the same time among the top importing countries, a considerable part of their export figure is comprised of processed products with an increased added value. As it is seen, besides being a producer in the sector, processing, and product diversification also provide countries important commercial advantages (FAO, 2020). When the processing level of Turkey's fisheries exports is examined, it is observed that unprocessed and less processed products with low added value are exported, and more processed products are imported. It is understood that the sector with the highest share of highly processed products in the exports of our country is in herbal products, and the highest unprocessed products are in fisheries (Yılmaz et al., 2018).

Turkey is an attractive country with its high foreign trade volume and growth rate among developing countries. In addition to its wide agricultural areas, its coastal and inland water facilities are the most important factors that shape the country's production model and comparative advantage structure (Kuşat, 2019). When the country's population and rapidly increasing world population are considered in terms of nutrition, fisheries production in addition to agricultural production gains importance. This study, it is aimed to measure both the competitiveness and export performance of the Turkish aquaculture sector against the leading countries of the world aquaculture sector. For this purpose, the sector's competitive power has been evaluated with the help of RCA, TBI, RMA, RTA, and RC indices.

Material and Methods

The main material of the study is the trade data obtained from the database of the International Trade Center (INTRACEN). In the study, the data set from the years between 2010 and 2019 is used. Apart from the data set, both Turkish and foreign literature reviews and statistics were also used in the study. The results of the Balasa Revealed Comparative Advantage-RCA index, Vollrath Relative Export Advantage-RXA index, Relative Trade Advantage-RTA index, Relative Competitiveness-RC index, Relative Import Advantage-RMA index, and Trade Balance Index-TBI index are presented in tables. Several indices are used in international trade to measure competitive power. The most commonly used indices are Balassa and Vollrath indices. (Hinloopen & Marrewijk, 2001; Welch & Lyford, 2007; Tao & Fu, 2007; Fertõ, 2008; Serin & Civan, 2008; Bojnec & Fertõ, 2012; Erkan et al., 2015; Terin et al., 2018). This is the reason to use them in this study as well. Developed by Balassa (1965) this index is called the Balassa index. In this index, the share of an industry in the country's total exports is calculated and it is proportioned to the share of world exports in the same industry in the total world (Atik, 2005; Akdağ, 2013). Revealed Comparative Advantage-RCA index is an index used to measure specialization in international trade and is widely accepted in the literature (Kanaka & Chinadurai, 2012; Pilinkiene, 2014; Torok & Jambor, 2016). RCA index is used in studies to determine the strong and weak export sectors (Aiginger, 2000; Bojnec & Fertõ, 2007; Terin & Yavuz, 2018). The purpose is to determine whether the country has a comparative advantage, rather than identifying the reasons underlying comparative advantage (Çakmak, 2005). Balassa's RCA index is formulated as follows (Equation 1):

$$RCA_{ij} = \left[\left(\frac{X_{ij}}{X_i}\right)/\left(\frac{X_{wj}}{X_w}\right)\right] \quad (1)$$

Here RCA_{ij}, X_{ij}, X_i, X_{wj}, and X_w indicate respectively; the revealed comparative advantage index for sector '*j*' of country '*i*', the export of sector '*j*' of country '*i*', the total export of country '*i*', the world export of sector '*j*', and the total world export. RCA index takes a value between 0 and ∞. If the index value is greater than 1, it indicates that the country for the calculation of competitiveness has a comparative advantage. If it is less than 1, then it shows that the country has no competitive power and has no comparative advantage (Hinloopen & Marrewijk, 2001; Havrila & Gunawardana, 2003; Esmaeili, 2014).

Developed as an alternative to the RCA index of Balassa, Vollrath's index is commonly used. According to Vollrath, import values should be taken into account together with exports in the calculation of the index. Thus, Vollrath developed 3 methods of measurement as an alternative to the index of Balassa. The first of the measurements is the Relative Export Advantage (RXA) index. When the RCA and RXA index formulas are examined, it is seen that it is RCA = RXA.

Relative Export Advantage index can be defined as the ratio of the export share of any country in the world markets to the share of all other goods in the world export in a given product (Atik, 2005). This feature of the index enables the countries and goods being measured to be excluded when calculating the total export (world) thereby avoiding them to be calculated twice (Fertõ & Hubbard, 2003; Çakmak, 2005; Altay & Gürpınar, 2008). The second method of measurement of Vollrath is Relative Trade Advantage (RTA) which is calculated as the difference between Relative Export Advantage (RXA) and Relative Import Advantage (RMA). The third method is the Relative Competitiveness index. If the RXA index is greater than 1, it indicates that the sector in question has a competitive advantage (Utkulu & Seymen, 2004). According to Vollrath, if

the RMA value is less than 1, the sector in question has a competitive advantage, and RC indices show comparative advantage if they are positive, and negative values indicate comparative disadvantage (Vollrath, 1991; Frohberg & Hartmann, 1997; Akhtar et al., 2013; Bashimov, 2016). These indices are formulated as follows (Equations 2-5):

$$RXA_{ij} = \frac{\left({}^{X_{ij}}/_{X_{nj}}\right)}{\left({}^{X_{ir}}/_{X_{nr}}\right)} \quad (2)$$

$$RMA_{ij} = \frac{\left({}^{M_{ij}}/_{M_{nj}}\right)}{\left({}^{M_{ir}}/_{M_{nr}}\right)} \quad (3)$$

$$RTA_{ij} = RXA_{ij} - RMA_{ij} \quad (4)$$

$$RC_{ij} = \ln\left(RXA_{ij}\right) - \ln\left(RMA_{ij}\right) \quad (5)$$

In these formulas, X = export, M = import, n = all remaining goods, and r = rest of the world. According to this; RTA = relative trade ij advantage in good i of country j RXA = relative export ij advantage in product i of country j RMA = relative import ij advantage in product i of country j RC = relative competitive advantage index in product i of country j.

Another index used in this study to determine the level of competitiveness is Trade Balance Index (TBI). TBI is used to determine whether a country is a net exporting or importing country in a given good and formulated as follows (Equation 6) (Lafay, 1992; Widodo, 2008).

$$TBI_{ij} = \frac{X_{ij} - M_{ij}}{X_{ij} + M_{ij}} \quad (6)$$

In this formula; TBI_{ij} shows the trade balance index in good j of country i. X_{ij} and M_{ij}, on the other hand, indicate the export and import in good j of country i. This index takes a value between -1 and +1. If $TBI_{ij} > 0$, the country is in the position of net exporter of the product in question. If $TBI_{ij} < 0$, the country is a net importer of the product in question (Widodo, 2008; Ullah & Kazuo, 2013; Topcu & Sümerli Sarıgül, 2015; Terin & Yavuz, 2019).

Results and Discussion

Considering the world fisheries and aquaculture production, 54% of the total production of 178 500 000 tons was obtained from fishing, while 46% was obtained from aquaculture. While aquaculture constituted 15% of the total production in the 1980s, it has increased significantly today (Table 1).

China takes first place in fish production from fishing with 14.6 million tons, followed by Indonesia with 7.2 million tons and Peru with 7.2 million tons (Figure 1).

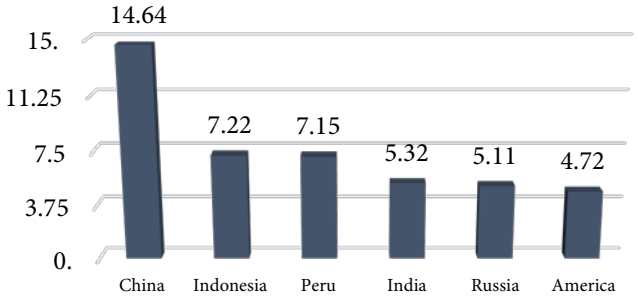

Figure 1. Top six global fisher capture producers (million tonnes)

Table 1. World fisheries and aquaculture production (million tonnes) (FAO, 2021)

Production	1986-1995	1996-2005	2006-2015	2016	2017	2018
Capture						
Inland	6.4	8.3	10.6	11.4	11.9	12.0
Marine	80.5	83.0	79.3	78.3	81.2	84.4
Total Capture	86.9	91.3	89.9	89.7	93.1	96.4
Aquaculture						
Inland	8.6	19.8	36.8	48	49.6	51.3
Marine	6.3	14.4	22.8	28.5	30.0	30.8
Total Aquaculture	14.9	34.2	59.6	76.5	79.6	82.1
Total World	101.8	125.5	149.5	166.2	172.7	178.5

Table 2. Competition index results in Turkey and leading countries in world aquaculture production (302)*

Country	Index	Year									
		2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
China	RXA	0.12	0.10	0.11	0.09	0.06	0.07	0.07	0.04	0.05	0.06
	TBI	0.25	0.25	0.08	0.08	-0.34	-0.35	-0.34	-0.56	-0.67	-0.66
	RMA	0.09	0.07	0.11	0.09	0.15	0.20	0.20	0.20	0.31	0.34
	RTA	0.04	0.03	0.00	0.00	-0.09	-0.13	-0.13	-0.15	-0.26	-0.29
	RC	0.34	0.39	0.00	-0.01	-0.93	-1.08	-1.05	-1.50	-1.84	-1.80
Indonesia	RXA	1.59	1.28	1.35	1.04	0.87	1.12	0.72	0.57	0.51	0.65
	TBI	0.97	0.98	1.00	1.00	0.96	0.87	0.63	0.51	0.46	0.52
	RMA	0.03	0.01	0.00	0.00	0.02	0.08	0.18	0.20	0.19	0.21
	RTA	1.57	1.27	1.34	1.04	0.85	1.03	0.54	0.36	0.32	0.44
	RC	4.12	4.63	6.39	6.01	3.82	2.61	1.39	1.03	0.99	1.15
India	RXA	0.15	0.18	0.30	0.27	0.31	0.28	0.24	0.15	0.13	0.18
	TBI	-0.12	-0.31	0.34	0.85	0.68	0.59	0.75	0.61	0.55	0.52
	RMA	0.12	0.24	0.09	0.02	0.04	0.05	0.03	0.03	0.02	0.04
	RTA	0.02	-0.06	0.21	0.25	0.27	0.23	0.22	0.13	0.10	0.14
	RC	0.16	-0.27	1.18	2.79	1.99	1.72	2.20	1.79	1.66	1.50
Peru	RXA	0.07	0.06	0.11	0.10	0.07	0.10	0.11	0.12	0.06	0.07
	TBI	-0.62	-0.55	-0.22	-0.25	-0.62	-0.73	-0.70	-0.70	-0.70	-0.51
	RMA	0.36	0.27	0.20	0.17	0.27	0.57	0.66	0.75	0.39	0.24
	TRTA	-0.29	-0.21	-0.09	-0.07	-0.20	-0.47	-0.55	-0.63	-0.33	-0.17
	RC	-1.68	-1.49	-0.59	-0.55	-1.39	-1.76	-1.78	-1.87	-1.86	-1.23
Russia	RXA	0.00	0.00	0.01	0.00	0.01	0.00	0.01	0.01	0.01	0.01
	TBI	-1.00	-1.00	-0.99	-1.00	-0.99	-0.98	-0.97	-0.98	-0.97	-0.97
	RMA	3.25	3.00	3.66	3.47	2.33	1.04	0.84	0.96	1.01	0.82
	RTA	-3.25	-3.00	-3.65	-3.46	-2.32	-1.04	-0.84	-0.96	-1.00	-0.81
	RC	-7.48	-7.78	-6.25	-6.78	-5.64	-5.41	-4.81	-5.19	-5.02	-4.77
Turkey	RXA	1.35	1.53	1.36	1.68	2.16	2.45	2.29	2.44	2.45	2.60
	TBI	0.68	0.63	0.57	0.69	0.74	0.69	0.80	0.82	0.84	0.86
	RMA	0.16	0.20	0.26	0.20	0.22	0.32	0.19	0.17	0.17	0.18
	RTA	1.18	1.33	1.11	1.48	1.94	2.13	2.10	2.27	2.28	2.42
	RC	2.11	2.03	1.67	2.15	2.28	2.03	2.48	2.68	2.69	2.70

Note: *302: Calculated by authors (Fish, fresh or chilled)

Table 3. Competition index results in Turkey and leading countries in world aquaculture production (303)*

Country	Index	Year									
		2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
China	RXA	0.77	0.93	0.91	0.89	0.89	0.89	0.95	0.88	0.89	0.85
	TBI	0.92	0.94	0.92	0.91	0.90	0.90	0.89	0.85	0.73	0.63
	RMA	0.03	0.03	0.04	0.05	0.06	0.06	0.07	0.09	0.16	0.24
	RTA	0.74	0.90	0.88	0.84	0.84	0.83	0.88	0.79	0.73	0.62
	RC	3.16	3.51	3.17	2.89	2.76	2.65	2.59	2.27	1.73	1.28
Indonesia	RXA	1.76	1.72	2.25	2.10	1.85	1.76	1.98	2.05	1.63	1.90
	TBI	0.95	0.96	0.96	0.98	0.98	0.98	0.96	0.96	0.96	0.96
	RMA	0.05	0.03	0.04	0.02	0.02	0.02	0.04	0.04	0.03	0.04
	RTA	1.71	1.69	2.21	2.08	1.83	1.74	1.94	2.01	1.60	1.86
	RC	3.63	3.92	4.08	4.70	4.39	4.47	3.85	3.94	4.01	3.87
India	RXA	2.28	2.01	1.87	1.82	1.78	1.77	1.77	1.82	1.70	1.19
	TBI	1.00	0.99	0.99	0.99	0.99	0.98	0.97	0.97	0.97	0.93
	RMA	0.00	0.00	0.01	0.00	0.01	0.01	0.02	0.02	0.02	0.03
	RTA	2.28	2.00	1.86	1.82	1.77	1.76	1.75	1.81	1.69	1.16
	RC	6.85	6.37	5.69	5.93	5.29	4.96	4.34	4.67	4.69	3.75
Peru	RXA	0.68	1.98	2.25	1.44	1.33	1.02	1.32	1.63	1.28	1.72
	TBI	-0.49	0.45	0.53	0.01	0.03	-0.30	-0.12	0.08	-0.09	0.23
	RMA	2.12	0.82	0.72	1.40	1.16	1.64	1.66	1.56	1.65	1.15
	TRTA	-1.45	1.16	1.53	0.04	0.17	-0.61	-0.35	0.08	-0.37	0.57
	RC	-1.14	0.88	1.13	0.03	0.14	-0.47	-0.24	0.05	-0.25	0.40
Russia	RXA	3.60	2.87	3.05	3.27	3.03	4.43	5.06	4.23	4.40	4.41
	TBI	0.37	0.37	0.45	0.33	0.30	0.48	0.51	0.48	0.54	0.52
	RMA	2.56	1.98	1.79	2.77	2.90	2.92	2.58	2.36	2.49	2.46
	RTA	1.04	0.90	1.26	0.51	0.13	1.51	2.48	1.86	1.91	1.95
	RC	0.34	0.37	0.53	0.17	0.05	0.42	0.67	0.58	0.57	0.58
Turkey	RXA	0.26	0.31	0.30	0.43	0.40	0.34	0.32	0.33	0.44	0.53
	TBI	-0.31	-0.19	-0.11	-0.01	0.02	-0.27	-0.12	-0.24	0.02	0.22
	RMA	0.27	0.22	0.23	0.27	0.26	0.40	0.29	0.36	0.32	0.29
	RTA	-0.01	0.08	0.07	0.16	0.15	-0.06	0.03	-0.03	0.12	0.24
	RC	-0.04	0.32	0.28	0.47	0.44	-0.17	0.09	-0.09	0.33	0.59

Note: *303: Calculated by authors (Frozen fish)

Table 4. Competition index results in Turkey and leading countries in world aquaculture production (304)*

Country	Index	Year									
		2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
China	RXA	2.02	2.02	1.91	1.79	1.70	1.54	1.49	1.47	1.38	1.30
	TBI	0.76	0.68	0.58	0.50	0.48	0.40	0.33	0.27	0.01	-0.24
	RMA	0.31	0.40	0.53	0.63	0.68	0.85	0.96	1.00	1.55	2.47
	RTA	1.72	1.62	1.37	1.16	1.02	0.68	0.53	0.47	-0.17	-1.17
	RC	1.89	1.62	1.27	1.04	0.91	0.59	0.44	0.38	-0.11	-0.64
Indonesia	RXA	1.40	1.30	2.06	1.93	2.00	2.30	2.20	2.00	2.21	2.44
	TBI	0.83	0.70	0.75	0.70	0.73	0.79	0.72	0.72	0.74	0.71
	RMA	0.15	0.25	0.28	0.31	0.29	0.27	0.38	0.35	0.31	0.38
	RTA	1.26	1.04	1.79	1.61	1.71	2.03	1.82	1.65	1.89	2.05
	RC	2.26	1.63	2.01	1.82	1.94	2.13	1.76	1.76	1.95	1.85
India	RXA	0.46	0.47	0.64	0.40	0.36	0.48	0.45	0.58	0.64	0.57
	TBI	0.93	0.89	0.90	0.83	0.77	0.78	0.78	0.84	0.83	0.81
	RMA	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.03	0.04	0.04
	RTA	0.45	0.45	0.63	0.37	0.33	0.44	0.41	0.54	0.60	0.53
	RC	3.84	3.31	3.55	2.74	2.47	2.52	2.43	2.87	2.86	2.67
Peru	RXA	1.79	1.82	2.57	1.91	2.46	3.06	2.81	2.28	2.33	1.87
	TBI	0.80	0.74	0.75	0.67	0.64	0.76	0.76	0.68	0.70	0.63
	RMA	0.22	0.33	0.38	0.35	0.48	0.34	0.37	0.47	0.44	0.44
	TRTA	1.57	1.50	2.19	1.56	1.99	2.72	2.45	1.81	1.89	1.43
	RC	2.08	1.72	1.90	1.69	1.65	2.19	2.04	1.58	1.66	1.45
Russia	RXA	0.41	0.41	0.30	0.48	0.57	0.69	0.72	0.74	0.66	0.70
	TBI	-0.17	-0.16	-0.33	-0.03	0.05	0.23	0.26	0.35	0.25	0.27
	RMA	0.95	0.92	0.92	0.81	0.84	0.77	0.64	0.55	0.72	0.67
	RTA	-0.55	-0.51	-0.63	-0.32	-0.27	-0.08	0.08	0.19	-0.07	0.03
	RC	-0.85	-0.81	-1.13	-0.51	-0.39	-0.11	0.12	0.30	-0.10	0.05
Turkey	RXA	0.56	0.63	0.65	0.70	0.73	0.90	1.01	0.98	0.94	0.96
	TBI	0.48	0.54	0.60	0.62	0.68	0.69	0.78	0.74	0.82	0.78
	RMA	0.12	0.10	0.10	0.09	0.09	0.11	0.09	0.10	0.07	0.10
	RTA	0.44	0.53	0.55	0.61	0.65	0.79	0.92	0.88	0.87	0.86
	RC	1.57	1.81	1.90	2.03	2.14	2.12	2.47	2.33	2.61	2.29

Note: *304: Calculated by authors (Fish fillets and other fish meat)

Table 5. Competition index results in Turkey and leading countries in world aquaculture production (306)*

Country	Index	Year									
		2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
China	RXA	0.65	0.67	0.67	0.65	0.60	0.52	0.49	0.39	0.34	0.26
	TBI	0.38	0.25	0.13	0.10	0.10	-0.04	-0.11	-0.25	-0.55	-0.74
	RMA	0.33	0.43	0.59	0.65	0.64	0.84	0.92	0.96	1.48	2.20
	RTA	0.32	0.23	0.09	0.00	-0.04	-0.31	-0.43	-0.56	-1.14	-1.94
	RC	0.68	0.43	0.14	0.00	-0.06	-0.47	-0.63	-0.89	-1.48	-2.13
Indonesia	RXA	5.25	5.21	5.96	6.63	7.12	6.26	6.27	5.97	5.80	5.40
	TBI	0.95	0.91	0.90	0.91	0.93	0.93	0.91	0.92	0.90	0.88
	RMA	0.16	0.27	0.30	0.32	0.27	0.27	0.36	0.33	0.30	0.34
	RTA	5.10	4.94	5.65	6.31	6.85	5.99	5.90	5.64	5.50	5.06
	RC	3.51	2.95	2.97	3.03	3.28	3.15	2.85	2.90	2.96	2.76
India	RXA	4.23	5.02	5.81	7.23	8.34	8.40	8.52	9.58	9.20	9.17
	TBI	0.99	0.99	0.99	1.00	0.99	0.99	0.99	0.99	0.98	0.98
	RMA	0.01	0.01	0.02	0.01	0.02	0.03	0.03	0.04	0.06	0.05
	RTA	4.22	5.01	5.79	7.22	8.32	8.37	8.49	9.54	9.15	9.12
	RC	5.71	5.91	5.79	6.56	6.08	5.70	5.61	5.46	5.11	5.25
Peru	RXA	1.71	1.82	1.91	2.52	2.94	3.03	2.66	2.92	3.09	3.22
	TBI	0.92	0.91	0.92	0.90	0.88	0.55	0.71	0.55	0.47	0.61
	RMA	0.09	0.10	0.09	0.14	0.19	0.84	0.51	1.11	1.34	0.84
	TRTA	1.62	1.72	1.81	2.37	2.75	2.19	2.15	1.81	1.75	2.38
	RC	2.99	2.88	3.04	2.86	2.73	1.28	1.65	0.97	0.84	1.34
Russia	RXA	0.51	0.48	0.53	0.54	0.76	1.11	1.44	1.59	1.75	2.37
	TBI	0.04	0.01	0.09	0.01	0.22	0.59	0.53	0.60	0.64	0.71
	RMA	0.82	0.80	0.74	0.96	0.93	0.59	0.77	0.75	0.79	0.73
	RTA	-0.31	-0.32	-0.21	-0.41	-0.17	0.52	0.67	0.84	0.96	1.65
	RC	-0.48	-0.51	-0.33	-0.57	-0.21	0.63	0.62	0.75	0.80	1.18
Turkey	RXA	0.04	0.03	0.01	0.03	0.02	0.03	0.03	0.03	0.02	0.03
	TBI	0.24	-0.32	-0.48	-0.07	-0.25	-0.03	0.31	0.15	-0.03	0.04
	RMA	0.02	0.04	0.03	0.02	0.03	0.03	0.01	0.02	0.02	0.03
	RTA	0.03	0.00	-0.01	0.01	0.00	0.01	0.02	0.01	0.00	0.01
	RC	0.97	-0.08	-0.63	0.29	-0.18	0.21	0.85	0.54	0.15	0.22

Note: *306: Calculated by authors (Crustaceans, whether in the shell or not, live, fresh, chilled, frozen, dried, salted)

The results of the Relative Export Advantage Index (RXA), Relative Import Advantage Index (RMA), Relative Trade Advantage Index (RTA), Revealed Competitiveness Index (RC), and Trade Balance Index (TBI) used to measure competitiveness in the international world fish trade are given in the tables below. Since Balassa's RCA index values and Vollrath's RXA index values are the same, interpretations in the tables are made according to RXA index values instead of RCA. When the competition index values of fresh and chilled fish are examined in Table 2, it is seen that Turkey has a comparative advantage (RXA> 1) between 2010 and 2019. It is striking that Turkey is a net exporter according to the TBI index result (TBI> 0). RTA and RC index results were calculated as 1.82 and 2.28, respectively. According to these index results, Turkey has a comparative advantage in the international fresh and chilled fish trade. Kuşat (2019) found Turkey's competitive power in the fish trade high in his study. According to the average RXA index results, it has been determined that the leading countries in world seafood production do not have a comparative advantage. TBI index results are: China (-0.22), Indonesia (0.79), India (0.44), Peru (-0.56) and Russia (-0.98). According to these results, China, Peru, and Russia are net importers while Indonesia and India are exporters. When the RTA and RC index results of the same countries are examined, it can be said that countries other than India and Indonesia are disadvantageous countries in foreign trade of fresh and chilled fish.

When the competition indices of the leading countries in the world frozen fish production are examined, it can be said that especially Russia, Indonesia, India, and Peru have comparative advantages in the international frozen fish trade. Since the RXA values of China and Turkey are 0.88 and 0.36, respectively, it can be stated that these countries have no comparative advantages. When the TBI index values are examined, it can be said that while it is not certain for Peru (0.03), other countries except Turkey are net exporters. When we look at the index values of RTA (1.86 and 1.79) and RC (4.1 and 5.25) of Indonesia and India, it is seen that they have a very high competitive advantage.

When the competition index values of fish fillets and other fish meats (whether minced, fresh, chilled, or frozen) are examined in Table 4, it draws attention that between 2010 and 2019 Turkey did not have a comparative advantage in the trade of fish fillets and other fish meats, that it was an exporter and that it had a competitive advantage. Between those years, the average RXA, TBI, RTA, and RC index results for Turkey were calculated as 0.81, 0.67, 0.71, and 2.13, respectively. According to the index results of RXA, TBI, RTA, and RC, other countries except Russia have a comparative advantage in the international trade of fish fillets and other fish meats. The TBI and RMA index results reveal that countries other than Russia are net exporters in the international trade of fish fillets and other fish meats.

The study results showed that Indonesia, India, Peru, and Russia have a comparative advantage in the international shellfish trade (Crustaceans, whether in the shell or not, live, fresh, chilled, frozen, dried, salted) (Table 5). RXA, RMA and RC index results for these countries are as follows; Indonesia (5.99, 0.29, 3.01), India (7.55, 0.03, 5.72), Peru (2.58, 0.52, 2.06) and Russia (1.11, 0.79, 0.19). Here, Russia's RC index values fell to negative values in the first 5 years and took positive values in the following years. Considering the TBI results of these countries (0.92, 0.99, 0.74, 0.34), it can be said that they are a net exporter. Looking at the index results of China and Turkey, the RXA values are 0.52 and 0.02, respectively, and according to these results, it can be said that there is no competitive advantage in either country. When we look at the RMA and TBI values of these two countries, the following is seen: China (0.90, -0.07) and Turkey (0.02, -0.04). According to these values, it can be said that China and Turkey are exporters according to RMA value and importers according to the TBI index. In short, these two countries can be said to be disadvantageous countries in the shellfish trade.

Conclusion

As of 2018, 178.5 million tons of fisheries and aquaculture products are produced in the world. The major part of this production is carried out by China. Indonesia, Peru, India and Russia are the other countries that have an important share in production. In measuring the countries ranking first in the world seafood production and Turkey's competitive power in the international world walnut trade; Relative Export Advantage Index (RXA), Trade Balance Index (TBI), Relative Import Advantage Index (RMA), Relative Trade Advantage Index (RTA) and Relative Competitiveness Index (RC) were used. These index results show that even though countries have significant potential in production in some cases, they cannot get a say in international trade. The index results revealed that, although China has a significant share in the world's seafood production, it has no competitive advantage in products other than frozen fish and that it is an important importer. The results of the research have shown that Indonesia and India have a comparative advantage in the international aquaculture trade. It shows that these countries are net exporters in world aquaculture exports. Peru has shown that it has a comparative advantage in aquaculture trade, except for the sub-sector of

fresh or chilled fish. Although Turkey is not an important actor in world aquaculture production, it has made significant progress, especially in fresh and chilled fish production. According to the index results used in the measurement of competitiveness, it is seen that Turkey is advantageous in terms of foreign trade competition. Again, Turkey is an exporter in the seafood trade, except for Crustaceans. As a result, it was concluded that only a high amount of production is not enough to get a say in the world aquaculture trade.

Compliance With Ethical Standards

Authors' Contributions

Author AA designed the study. ND wrote the first draft of the manuscript, AA and ND read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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RESEARCH ARTICLE

Investigation of the relationship between bioluminescence and the production of α-amylase of the first bioluminescent *Vibrio gigantis* strains from Izmir Bay

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ABSTRACT

As an alternative to chemicals used in the industry, the use of enzymes is gradually increasing, with their high reaction specificity and their ability to show minimal by-product formation. In the detergent industry among the industrial areas where enzyme use is widespread; due to high washing temperatures, loss of activity of the detergent and high energy consumption, cold active enzymes that exhibit high catalytic activity at low temperatures and have the potential to save energy are noteworthy. As one of these enzymes, α-amylase is intensely produced by marine bioluminescent microorganisms that show optimum microbial activity at 20°C. However, since the enzyme production differs among microorganisms, selection of the most suitable microorganism to be used in production is very important. In this study, based on the idea that bioluminescence will benefit by facilitating the selection of microorganisms that will come to the fore for α-amylase production, the relationship between bioluminescence and the production of extracellular α-amylase enzyme of *Vibrio gigantis* strains, which were obtained from the sources of our country, were isolated from Izmir Bay and were determined to have a high rate of α-amylase production, and which was the first record in terms of bioluminescent properties, was investigated. Among 20 *V. gigantis* strains, 2 different microorganisms, which are thought to be more advantageous in terms of enzyme production and bioluminescence, were selected and the extracellular protein and α-amylase production amounts of these organisms as well as the amount of bioluminescence were measured. By evaluating the data obtained as a result of the studies carried out, further studies were carried out with 2 strains, S2W42 and FU-9 gill, which exhibit both low and high enzyme activity. Also, an inverse relationship was observed between α-amylase enzyme activity and bioluminescence. It has been determined that both microorganisms used are effective in α-amylase production and can be used as model organisms in cold active enzyme production. For this reason, it is thought that our study will shed light on comprehensive studies to be carried out in the relevant field.

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Introduction

Bioluminescence; is an interesting chemical reaction in which visible light is produced as a result of an enzyme-catalyzed oxidation reaction emitted by light-forming organisms (Ramesh & Mohanraju, 2015; Brodl et al., 2018). Although the benefit of bioluminescence, which has evolved independently dozens of times, cannot be determined for some species, in many cases; it is known that providing visual communication for behaviors such as attracting prey or intimidating predators provides a significant advantage to organisms in ecological terms (Fleiss & Sarkisyan, 2019). At the same time, it has also been reported that marine organism use bioluminescence for physiological events such as food recycling, concealment, mating and defense (Sharifian et al., 2018).

This feature, which attracts great attention in the world of science; is found in many phyla from fish to bacteria, bioluminescent organisms that persist in terrestrial, freshwater and marine ecosystems, and is observed in a large number of species (Nunes-Halldorson & Duran, 2003; Tanet et al., 2020). Such that; although the ability to emit light in the dark is observed in 800 genera containing approximately 10,000 species, it is thought that this number may actually be higher (Fleiss & Sarkisyan, 2019). Bioluminescent bacteria, which constitute an important group of these organisms, can survive in relation to marine animals by forming a symbiotic relationship with a host animal as well as being able to live in free state (Burtseva et al., 2020). In symbiotic forms, they are usually colonized in the light organs and intestines because they have more favorable growth conditions than open oceans and in the light of available information, most of the known bacterial luminous species show heterotrophic, copiotrophic and facultative anaerobic characteristics (Tanet et al., 2020).

Bioluminescence in bacteria occurs as a result of the reaction between molecular oxygen, a coenzyme (a mononucleotide flavin in a reduced state, $FMNH_2$) and a long chain aldehyde catalyzed by the luciferase enzyme. While it is seen that the products formed as a result of the reaction are FMN (in oxidized state), water and carboxylic acid, a photon is emitted during the reaction at the same time. While the oxidized flavin (FMN) and the carboxylic acid formed in the reaction are recycled by parallel reactions, the light production occurs continuously by living cells (Erzinger et al., 2018).

Looking at the literature; although interest in bioluminescent bacteria and light regulation has continued for a long time, the majority of studies have been limited to two model organisms, *Aliivibrio fischeri* and *Vibrio campbellii* (previously identified as *Beneckea* or *V. harveyi*). However; although bioluminescent bacteria cannot be limited to these two species, 25 bacterial species belonging to 5 genera have been discovered in the families of *Shewanellaceae* (*Shewanella*), *Enterobacteriaceae* (*Photorhabdus*) and *Vibrionaceae* (*Aliivibrio*, *Photobacterium* and *Vibrio*), which are three families of Gammaproteobacteria (Tanet et al., 2019).

Genetic, physiological and biochemical studies are carried out within the scope of bioluminescence, which has a wide application potential in different fields, and the progress achieved as a result of these studies sheds light on studies in ecological, medical and industrial fields (Nunes-Halldorson & Duran, 2003). Considering the industry as one of these fields; enzymes, which constitute an important alternative to chemicals used in many processes, are seen as substances with minimal risk potential in terms of environmental health with their reaction specificities and minimal by-product generation properties (Özcan & Çorbacı, 2018).

Industrial enzyme purification applications are generally focused on lipases, proteases and amylases (Gopinath et al., 2017). Amylases, which have a 30% share of the enzymes produced in the world today; it attracts attention with its potential applications in the textile, pharmaceutical, fermentation, detergent, food and paper industries (Özcan & Çorbacı, 2018). Amylases, which are starch-degrading enzymes, act on α-1-4 glycosidic bonds and are also called glycoside hydrolases, are widely distributed in living systems (Gopinath et al., 2017; Özcan & Çorbacı, 2018). In particular, enzymes known as cold active enzymes come to the fore with their high catalytic activity at low and medium temperatures, the need for low amounts of enzyme for efficient catalysis, and their economic advantage as a result of their energy-saving applications (Al-Maqtari et al., 2019).

In general terms, biologically active enzymes are obtained by animals, plants and microorganisms. But among these groups; it brings to the fore microorganisms that have the characteristics of being stable under extreme conditions due to their easy and high isolation, low cost and short time-consuming production and less harmfulness. In addition to these advantages, the production and expression of recombinant enzymes are easier in cases where the host cell is a microorganism (Gopinath et al., 2017). In this context; because of the aforementioned advantages, especially amylase-producing microorganisms adapted to low temperature and alkaline conditions are becoming popular for commercially important applications, especially in the detergent industries. For example, with respect to cold active amylase determined to be compatible with detergents, α-amylase was isolated from *Zunongwangia profunda*, a marine bacterium, and was found to be compatible with detergents. And then, it was determined

that the recombinant enzyme showed activity even at very low temperatures such as 0-5°C with the expression of the relevant gene in *Escherichia coli* (Al-Ghanayem & Joseph, 2020).

In the light of all this information, it is thought that bioluminescence, which has wide application areas as mentioned above, will have an effective contribution. In this study, it is aimed to reveal the relationship between extracellular α-amylase enzyme production and bioluminescence of *V. gigantis* strains, which were obtained from the sources of our country and were found to produce a high rate of extracellular α-amylase enzyme and at the same time recorded bioluminescence by us for the first time. With the data obtained as a result of our study, it was determined whether it is appropriate to use bioluminescence in pre-screening stages to select the strain that produces the highest amount of α-amylase enzyme from bioluminescent marine organisms, which are target organisms for the production of commercially important cold active α-amylase enzyme. As a result, it is thought that this study will facilitate strain selection in this context and shed light on further studies that can produce cold active α-amylase enzyme on an industrial scale.

Material and Methods

Activation of V. gigantis Strains

Twenty bioluminescent *V. gigantis* strains isolated from different seawater, sediment, and fish samples and collected from regions at discrete depths in the Gulf of Izmir were used for this study (Table 1). The bioluminescent bacteria identified by phenotypic and molecular methods (Ersoy Omeroglu, 2011; Ersoy Omeroglu & Karaboz, 2012; Ersoy Omeroglu et al., 2014) were streaked onto a Seawater Complete Agar (SWC) medium to obtain a single colony. After checking the purity of the strains, they were grown in a liquid SWC medium (Liu et al., 2003). Following the inoculation procedures, the strains were incubated at 20°C for 17 hours (Ersoy Omeroglu & Karaboz, 2012). For the activation and bioluminescence controls (Figure 1), the microorganisms showing the most emission were determined by comparing the biological luminescence observed on the plates belonging to the strains.

Table 1. Some properties of bioluminescent *V. gigantis* strains used in the present study (Ersoy Omeroglu, 2011; Ersoy Omeroglu & Karaboz, 2012; Ersoy Omeroglu et al., 2014).

Strain No	Strain	Isolation Date	Accession Number	Source	Coordinate	Depth (m)
1	SW15	30 March 2007	JF412215	Seawater	38°29'03" N – 26°47'05"E	0-15
2	SWPort	30 March 2007	JF412216	Seawater	38°27'22" N – 27°09'65"E	
4	Selü25	30 March 2007	JF412217	Sediment	38°23'50" N – 26°39'00"E	
6	S2W42	22 January 2008	JF412218	Seawater	38°24'58" N – 26°56'88"E	
7	S2W9	22 January 2008	JF412219	Seawater	38°34'99" N – 26°39'00"E	
8	S3W46	17 April 2008	JF412220	Seawater	38°26'70" N – 27°06'10"E	
9	S3W28	17 April 2008	JF412221	Seawater	38°23'50" N – 26°55'00"E	
10	S3W2	17 April 2008	JF412222	Seawater	38°40'90" N – 26°34'90"E	
12	Se2Lü48	22 January 2008	JF412223	Sediment	38°24'75" N – 26°58'90"E	
15	Se3Lü25	22 January 2008	JF412224	Sediment	38°23'50" N – 26°39'00"E	
19	FU-10 internal	30 March 2007	JF412225	*Mullus barbatus* (internal area)	Between 38°37'00"N – 26°42'20"E and 38°37'45"N – 26°43'30"E	67
20	FU-9 gill	30 March 2007	JF412226	*Diplodus annularis* (gill)		
31	E-14 gill	17 April 2008	JF412227	*Lepidotrigla cavillone* (gill)	Between 38°34'40"N – 26°46'10"E and 38°33'45"N-26°46'55"E	42-44
32	E-16 surface	17 April 2008	JF412228	*Boops boops* (surface)		
33	E-15 surface	17 April 2008	JF412229	*D. annularis* (surface)		
34	E-10 gill	17 April 2008	JF412230	*Citharus linguatula* (gill)		
36	E-11 internal	17 April 2008	JF412231	*Arnoglossus laterna* (internal area)		
44	H-3 surface	06 August 2008	JF412232	*Merluccius merluccius* (surface)		
49	H-2 gut	06 August 2008	JF412233	*D. annularis* (intestine contents)		
50	H-16 gill	06 August 2008	JF412234	*B. boops* (gill)		

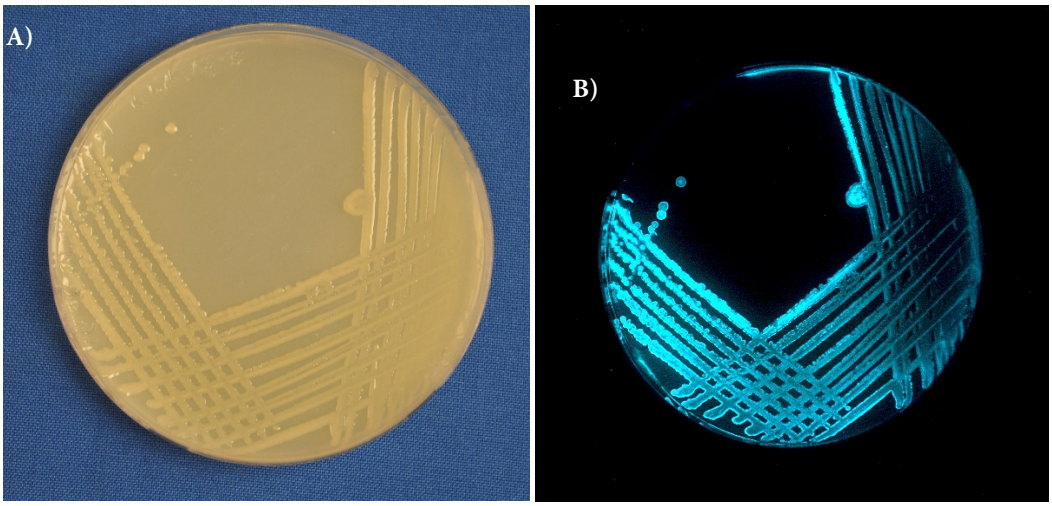

Figure 1. Streak plate images of *V. gigantis* SW15 strain. A) In the light; B) In the dark

Determination of Extracellular α-Amylase Production

Activated *V. gigantis* strains were inoculated into Starch Agar (SA) medium to determine their extracellular enzyme production and incubated for 17 hours at 20°C. At the end of the incubation period, gram iodide was dropped on the plates and left for 10 minutes incubation in order to observe the zone diameters. After the incubation period, the light-colored zone diameters observed around the colony and considered as positive results were measured (Özcan & Çorbacı, 2018).

By evaluating the amount of biological luminescence observed in the petri dishes during the activation phase and the zone diameters obtained in the enzyme production trials, the microorganisms with the highest bioluminescence and zone diameter formation were determined. The strain number 6 *V. gigantis* (S2W42) isolated from the marine environment and the strain number 20 *V. gigantis* (FU-9 gill) isolated from the gill of *D. annularis*, which were thought to have an advantage in terms of the hypothesis of the study, were selected and used in the other stages of the study. At the same time, while these two organisms were selected; their isolation from different regions has also been taken into account in terms of providing diversity.

Extracellular Protein Quantification by Bradford Method

Protein quantification was performed using the Bradford method for *V. gigantis* (S2W42) and *V. gigantis* (FU-9 gill). In order to obtain extracellular proteins, the nutrient medium was centrifuged at 10,000 rpm for 5 minutes and the supernatant obtained was used as a protein sample in the next steps. The standard was set containing 15 different concentrations of Bovine Serum Albumin (BSA), ranging from 0.02 to 2 mg/mL. In order to prevent all organic contamination that may cause erroneous results in enzyme amount measurements, all glass materials that may be required in the experiment were acidified and cleaned with distilled water before application. It was then dried in a Pasteur oven. After the determined concentrations and samples were prepared and reagent was added to them, color variations were observed at the end of incubation. After the incubation period, extracellular protein was determined using the spectrophotometric method (Yaşa & Çadırcı, 2007). In this study, standards and samples have been studied in duplicate.

Detection of Reducing Sugar

Dinitrosalicic acid (DNS) was used as a color reagent to detect the reducing sugar that will be formed by hydrolysis as a result of the interaction of starch with amylase. All glass materials used were acidified, cleaned with distilled water, dried in the Pasteur oven and used. Thus, the possibility of protein contamination was prevented. The organisms to be used were inoculated into Starch Broth (SB) medium and incubated at 20°C for 17 hours. Maltose standard solution (4 mg/mL stock solution), phosphate buffer, starch solution and DNS as reagent were prepared to be used in assay. From 4 mg/mL stock maltose solution, 7 standards were determined from 0.025 to 0.9 mg/mL. The determined concentrations and samples were prepared, vortexed and then incubated at 25°C for 5 minutes. At the end of the incubation, the DNS reagent was added to the tubes, mixed with vortex, kept in boiling water for 10 minutes and then allowed to cool. After the tubes were cooled, they were taken into polystyrene cuvettes and spectrophotometrically measured at 546 nm wavelength (Yaşa & Çadırcı, 2007).

Bioluminometric Measurements

Bacterial inoculations were made by preparing SB and Nutrient Broth (NB) with 70% natural seawater to be used in measurements with a white 96-well plates. The strains were incubated at 20°C for 17 hours in order to obtain bacteria in the logarithmic phase. Following the incubation period, transfer of the medium prepared to the wells determined on the microplate and microorganism inoculations were performed. To each well, 180 µL of the medium was added and then 20 µL of bacterial suspension with a turbidity equivalent to 1.0 McFarland (Osawa et al., 1997; Musa et al., 2008; Ersoy Omeroglu et al., 2014) was transferred into each well. *V. gigantis* (S2W42) and *V. gigantis* (FU-9 gill) inoculated to SB media and in addition, both strains were inoculated in the same way in NB media containing 70% natural seawater. These groups formed PK1 and PK2, respectively. Non-inoculated SB and NB media were also included in the measurements and designed as negative controls and evaluated as NK1 and NK2, respectively. 17 hours and 41 hours after the completion of the inoculation in the determined wells in white 96-well plates, two measurements were made with a bioluminometer, the unit of which is Relative Light Unit (RLU) (Tanet et al., 2019). In order to increase the reliability of the measurement results, the inoculations were made on a microplate in 3 repetitions and the average value was calculated in the measurement results.

Results

Detection of Extracellular α-Amylase Production

The measurement results of the zone diameters resulting from the enzyme activities of bioluminescent *V. gigantis* strains were given in Table 2. When the formed zone diameters are evaluated; it is seen that SWPort, S3W46 and S3W28 strains obtained from different locations from sea water stand out as the species with the highest activity. The lowest activity result, on the other hand, was obtained from surface swabs (E-15 surface) of *D. annularis* as a result of sampling in the spring season. However, it was determined that extracellular α-amylase enzyme production was not performed in two of the 20 strains studied (FU-10 internal and E-11 internal) because no zone formation was observed. Considering their characteristics such as the amount of bioluminescence (Table 2), growth rate and enzyme activity, 2 strains were determined at this stage and studies were continued with these 2 strains. In the last step of the selection, 2 strains with both low and high enzyme activity were decided and further studies were carried out with S2W42 and FU-9 gill.

Table 2. Observed zone diameters based on extracellular α-amylase production and the amount of bioluminescence by *V. gigantis* strains

Strain No	Strain	Zone Diameter (mm)	Bioluminescence
1	SW15	5.0	+[c]
2	SWPort	8.0	++
4	Selü25	5.0	++[b]
6	S2W42	5.0	+++[a]
7	S2W9	7.0	+
8	S3W46	8.0	++
9	S3W28	8.0	+
10	S3W2	4.0	+++
12	Se2Lü48	5.0	+
15	Se3Lü25	5.0	++
19	FU-10 internal	*	+++
20	FU-9 gill	7.0	+++
31	E-14 gill	5.0	+++
32	E-16 surface	6.0	+
33	E-15 surface	2.0	+++
34	E-10 gill	6.0	+
36	E-11 internal	*	+
44	H-3 surface	5.0	++
49	H-2 gut	6.0	++
50	H-16 gill	6.0	++

Note: *: The zone is not detected. [a]: Good bioluminescence; [b]: Average bioluminescence; [c]: Poor bioluminescence.

Extracellular Protein Quantification

As a result of the spectrophotometric measurements performed as specified in the method, a standard graphic was created and its reliability was checked based on the R^2 value. Protein calculation was performed by obtaining the necessary formulation. The standard graphic and the amount of protein were given in Figure 2 and Table 3, respectively.

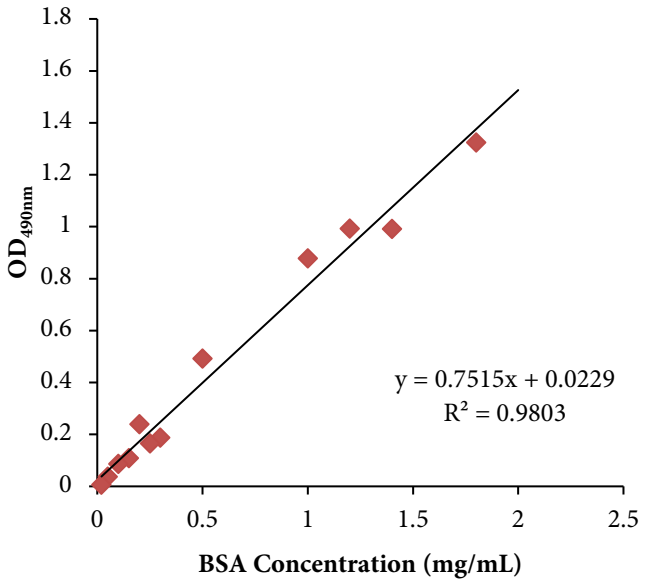

Figure 2. Bradford assay standard curve for the absorbance of BSA concentration

It has been determined that the amount of extracellular protein produced by *V. gigantis* strain FU9 (gill) obtained from the intestinal contents of *D. annularis* is higher than the amount of protein obtained from S2W42, the strain obtained from sea water (Table 3).

Detection of Reducing Sugar

The maltose standard graph obtained as a result of the measurements performed spectrophotometrically at 546 nm wavelength was shown in Figure 3. With the formulation obtained on the graphic, the amount of α-amylase and the values of specific activity were calculated and given in Table 3.

Extracellular enzyme amounts and activities were calculated using the graphic created based on the determination of reducing sugar. Although the amount of extracellular protein produced by S2W42 was less, it was found that the amount and activity of α-amylase produced as expected was higher. Although, as a result of the experiment performed on agar plates, a zone diameter of 5 mm was obtained for S2W42 and 7 mm for FU9 (gill); as a result of the activity determination performed by spectrophotometric method, it was determined that the opposite situation was in question. When we compared these two strains in terms of the amount of α-amylase produced, it was revealed that there was a 5.5% difference. The same ratio was observed in enzyme activity values. The difference of 35.6% in the amount of extracellular protein produced by bioluminescent *V. gigantis* strains showed its effect in specific activity. It was determined that the S2W42 strain had 39% higher specific activity (Table 3).

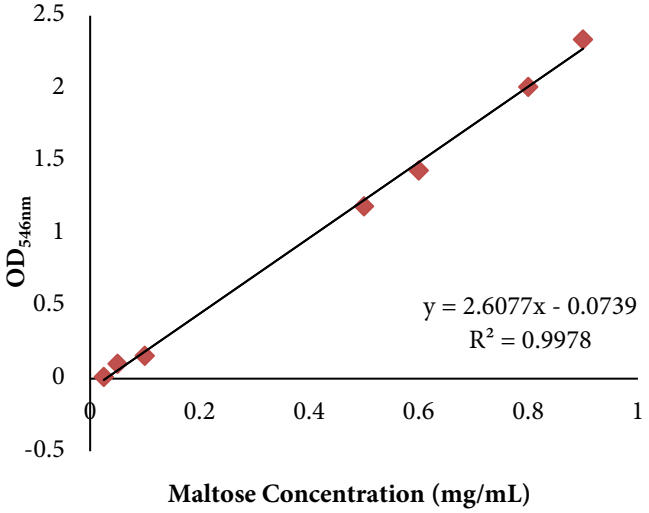

Figure 3. Standard curve for maltose determination assay

Table 3. Extracellular protein amount, α-amylase amount, α-amylase enzyme activity, specific activity and bioluminescence values of *V. gigantis* strains

Strain No	Strain	Extracellular Protein Amount (mg/mL)	α-amylase Amount (mg/mL)	α-amylase Enzyme Activity (IU/mL)	Specific Activity (IU/mg)	Bioluminescence (17th hour) (RLU)
6	**S2W42**	0.0551	0.0586	0.2344	4.254	11.5
20	**FU-9 (gill)**	0.0856	0.0554	0.2216	2.5887	27.3

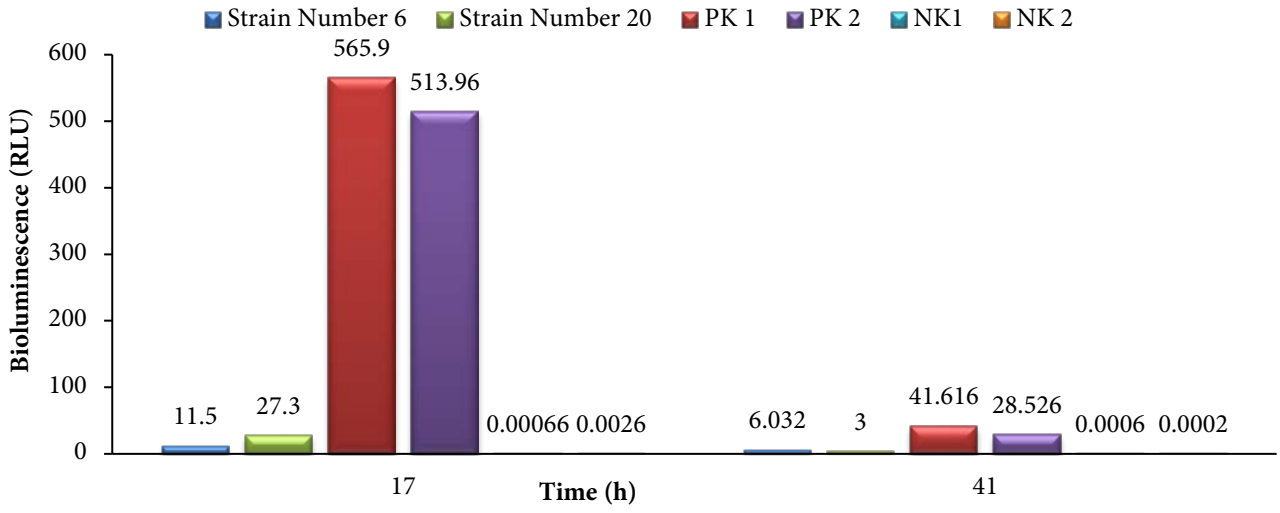

Figure 4. 17th and 41th hours measurement results performed with luminometer. PK1: Positive control 1 (NB+*V.gigantis* (S2W42) strain number 6), PK2: Positive control 2 (NB+*V.gigantis* (FU-9 gill) strain number 20), NK1: Negative control 1 (SB), NK2: Negative control 2 (NB)

Bioluminescence Measurement

The results of the measurements performed with the bioluminometer at 17 hours after inoculation and 24 hours after the first measurement are shown in Figure 4.

It was observed that there is an inverse relationship between α-amylase enzyme activity and bioluminescence in the bioluminescent strains selected within the scope of the study. As a common feature for both strains, it was determined that there was a significant decrease in the amount of bioluminescence at the 41st hour of incubation both in the experimental environment and in the positive controls. Especially, it was determined that the longer the incubation time had a more negative effect on FU9 (gill) (Figure 4).

Discussion

Amylases as extracellular enzymes that hydrolyze the starch constitute a significant 30% of the world enzyme market. Cold active amylases are suitable for application in detergents for cold washing purposes, as well as in various industrial areas such as food industry, textile, paper, molecular, pharmaceutical, bioremediation, alcohol production and biofuels. They come into prominence with their low energy consumption due to their active activity at low temperatures and economic benefits (Arabacı & Arıkan, 2018; Al-Maqtari et al., 2019).

Considering detergents that have an important share in the industrial field, it is thought that it is possible to achieve an environmentally friendly approach by using enzymes compatible with detergents instead of harmful chemicals as additives and The addition of α-amylase enzyme to detergents to effectively remove starch-based dirt and stains is an example of applications in this context. In this direction, the isolation and feasibility studies of cold active amylases to be used as detergent additives have gained continuity (Al-Ghanayem & Joseph, 2020) and therefore, microorganisms that can be a source of cold active enzymes are very valuable (Arabacı & Arıkan, 2018).

Studies on amylase obtained from bacteria, yeast and fungi continue in industrial fields and scientific research. Bacteria that are among the groups of organisms that show amylase production come to the fore because they give faster results and require less cost, and they have a wide application potential that includes the production of recombinant enzymes for genetic engineering studies. But, the amylase production level varies from microorganism to microorganism even between the same genus, species and strains (Gopinath et al., 2017). Therefore, organism selection constitutes an important step in enzyme production.

Based on this point, in order to be used bioluminescence in the selection of the advantageous strain in terms of enzyme production; within the scope of our study, 20 different bioluminescent strains of *V. gigantis* were examined in terms of biological luminescence and extracellular α-amylase production activities. As a result of the pre-screening, 2 bioluminescent strains that showed a high amount of biological radiation and obtained a high zone diameter due to extracellular enzyme activity, so that it was seen to be advantageous by comparing both criteria. Trials were continued to explain the relationship between bioluminescent and extracellular enzyme activity with the selected strain number 6 *V. gigantis* (S2W42) and strain number 20 *V. gigantis* (FU-9 gill). Such that; for this study, our initial research hypothesis was that as bioluminescence increases, extracellular α-amylase activity will increase, so the organism with high bioluminescence will show a high level of enzymatic activity. Thus; based on this hypothesis, and by identifying the organisms that can be used in the production of cold active α-amylase enzyme, the first step of producing α-amylase enzyme, the pre-screening step, was considered to be completed.

If the data obtained as a result of our study are evaluated, differences were observed in both organisms that did not comply with our hypothesis. First, when we look at the data of strain number 6 *V. gigantis* (S2W42), when the organism is inoculated into SB medium other than SWC agar, which is the closest nutrient medium to its own environment, there was a decrease in its bioluminescence, but an increase in enzyme production.

Secondly; when the data of *V. gigantis* (FU-9 gill) are examined, it was determined that its bioluminescence was 3 times higher than *V. gigantis* (S2W42) by showing the opposite characteristics when they were inoculated into SB medium instead of SWC agar, which is the medium closest to its own growth medium. Considering that bioluminescent microorganisms need to use 20% of their metabolic energy to perform bioluminescent activity; it is concluded that when strain number 6 *V. gigantis* (S2W42) is inoculated in a different environment, it spends most of its energy on enzyme production, but strain number 20 *V. gigantis* (FU-9 gill) consumes its energy in bioluminescence when under the same conditions.

It is thought that there are many reasons that will produce these results. The first reason is that the organisms are only 80% similar as stated by Ersoy Omeroglu & Karaboz (2012). This situation is in line with the knowledge in the literature that there is a difference in enzyme production even between species and strains among organisms (Gopinath et al., 2017). Another reason is that the isolation sources of organisms are different.

Strain number 6 *V. gigantis* (S2W42) was isolated from sea water. Therefore, it is thought that the bioluminescence is high in the SWC agar medium, which is the closest to its own habitat, but there is a decrease in its bioluminescence and an increase in enzyme production when taken to another environment. The strain number 20 *V. gigantis* (FU-9 gill) differs in that it is isolated from the gill of *D. annularis*.

Another possibility that may cause this difference is that reproductive temperatures differ between bioluminescent microorganisms. It was revealed by Ersoy Omeroglu & Karaboz (2012) that strain number 6 *V. gigantis* (S2W42) reproduced at 30°C and strain number 20 *V. gigantis* (FU-9 gill) at 27°C. At the same time, considering the temperature grouping of microorganisms from which cold-active enzymes are isolated, it is stated in the literature that most of the cold-active enzymes are isolated from psychrophil and psychlotolerant microorganisms, but also from microorganisms with mesophilic and even thermophilic properties (Santiago et al., 2016). According to an information based on the literature; the cold active α-amylase enzyme was isolated from *Bacillus subtilis* N8 at 15°C and pH 10, and the enzyme showed the highest activity at 25°C and remained stable at pH 8. It also showed a very high stability of 96% in a wide temperature range of 10-40°C (Al-Ghanayem & Joseph, 2020). With this information, the idea of using cold active enzymes for the production of bioluminescent bacteria used in our study is supported due to the similarity of the temperature values at which they reproduce. In terms of the convenience it will provide in the isolation and subsequent examination studies, it is thought that it would be advantageous to use bioluminescent bacteria used in this study as a model instead of extreme organisms that survive at low temperatures.

As a result of this study, it has been proven that both bioluminescent *V. gigantis* strains produce α-amylase and can be used as model organisms in cold active enzyme production. The data obtained and the approaches put forward will form the basis for future studies to be carried out in the relevant field.

Conclusion

Nowadays, concerns about harmful chemicals used in the industry are increasing and efforts are being made to replace these chemicals with enzymes that provide advantage with their high specificity and low by-product formation. The high temperatures used in the detergent industry, which has a very wide market, reduced detergent effectiveness and high temperatures negatively affected energy use, which required alternative solutions to be found. Cold active enzymes come to the fore with their high activity at low temperatures. Therefore, obtaining α-amylase, one of the cold active enzymes, has gained importance. Microorganisms, especially bacteria, constitute an important source for the α-amylase enzyme, with their advantages such as high-speed growth and low cost. At this point, it is thought that bioluminescence as a method to be used in the selection of organisms that differ in enzyme production will accelerate the related studies.

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Compliance With Ethical Standards

Authors' Contributions

EEO designed the study. EEO and AB wrote the manuscript. EEO, AB and BST performed laboratorial work. EEO critically revised the manuscript. All authors read and approved the final version of the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report.

Ethical Approval

For this type of study, formal consent is not required.

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RESEARCH ARTICLE

Growth and survival performance of smooth scallop (*Flexopecten glaber* Linnaeus, 1758) at different depths in the Aegean Sea

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ABSTRACT

This study was conducted between July 2016 and 2017 to determine the growth and survival rates of the smooth scallop *Flexopecten glaber* spats in Urla Karantina Island. The sea water temperature was determined as 21.56±6.33°C, 21.1±6.40°C and 20.87±6.35°C at 2, 4 and 6 m depths, respectively. Salinity values varied between 36 and 38.19 PSU in the region. The highest chlorophyll-*a* value was determined as 8.95 µg l^{-1} in August at 2m depth and 1.65 µg l^{-1} as the lowest at 4 m depth in January. Average values of total particulate matter amount were calculated as 4.41±1.86 mg l^{-1}, 5.09±1.88 mg l^{-1} and 5.47±1.89 mg l^{-1} at 2, 4 and 6m depth, respectively. Scallop spats with an average height of 8.26±1.55 mm were measured at the beginning of the study. The heights of the smooth scallop spats, which were placed at 2m, 4m and 6m depths in the study area, were 42.6±1.11 mm, 41.53±12.85 mm and 41.57±1.64 mm and their weights were measured as 12.71±0.89 g, 12.85±0.53 g and 12.82±1.00 g, respectively. While the survival rate was 53% placed at 2m depth in the study area, the lowest survival rate was found as 37% for the spats grown at 6m depth. The result showed that the mean values of height at the surface depth (2m) were more significant than those at the other depths (4m and 6m). However, there were no statistically significant differences between the depths and specific growth rate (SGR) for height and weight ($p>0.05$). But SGRh and SGRw values at each depth showed statistically significant differences between months ($p<0.05$).

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Introduction

About 40 species of scallops belong to the Pectinidae family, suitable for human consumption and used commercially (Waller, 1991; Minchin, 2003). The smooth scallop (*Flexopecten glaber* Linnaeus, 1758) is an invertebrate bivalve from the Mollusca phylum and is widely spread in the Mediterranean (Poutiers, 1987; Mattei & Pellizzato, 1996; Zenetos, 1996; Tsotsios et al., 2016). This species is one of the high market value bivalve species for the aquaculture industry (Tsotsios et al., 2016). Aquaculture is the fastest growing food production area in the world (FAO, 2020), especially molluscs are an important source of nutritious animal protein. Environmental changes and heavy fishing pressure have led to

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the destruction of fish and scallop populations in many countries (Strand & Vølstad, 1997; Stotz & Mendo, 2001). In some countries, the population of scallop has decreased; because of this, the culture of this species is aimed through stock configuration and conservation programs (Tettelbach et al., 2002; Drummond, 2004).

The bivalve stock supply for production is usually made by collecting spat from nature with polyethylene net bags (Kurtay et al., 2018; Yigitkurt et al., 2020). The culture methods of scallop are the bottom culture, floating cages, sink cages, and suspended methods (Paul et al., 1981; Ventilla, 1982; Roman & Acosta, 1991; Slater, 2005). The preferred culture production method varies according to the location of the cultivation area (Leavitt, 2010).

Bivalve culture in Turkey is limited to the Mediterranean mussel. *F. glaber* has a potential product for aquaculture in Turkey (Vural & Acarli, 2019; Vural & Acarli, 2021) due to its nutritional quality. In addition, this species has high growth and survival performance, early maturation and intensive breeding (Tsotsios et al., 2016).

This study aimed to collect *F. glaber* spats to find the differences between growth and survival rates at different depths and ensure the culture's sustainability by determining the environmental effects on production.

Material and Methods

Study Site

This study was carried out at the coast of Urla Karantina Island in the Aegean Sea which is located at 38°22'44" N and 26°47'12" E (Figure 1).

Figure 1. Study area at Urla Karantina Island

Collector and Suspended Culture System

The scallop spats were obtained between July 2016 and August 2016 with spat collectors designed by connecting 20 collectors prepared using polyethylene net bags with a size of 100×29 cm and a mesh size of 5×4 mm (Figure 2a).

Spats were cultured from August 2016 to July 2017. The suspended culture system was designed with a diameter of 20 cm, a thickness of 5 cm, and a mesh size of 5×5 mm on both sides. The collected spats were placed in three depth culture systems as 90 individuals in each system in 3 repetitions and were cultivated by hanging at different depths (2 m, 4 m, and 6 m) (Figure 2b). According to the growth of the scallop, the mesh size of the net used was increased periodically.

Height and Weight Measurements

The shell height of the scallops was measured from the maximum height between the dorsal (hinge) and ventral edge using a Mitutoyo digital caliper, monthly. The samples were weighed using a digital balance (Sartorius, GW3202-O CE).

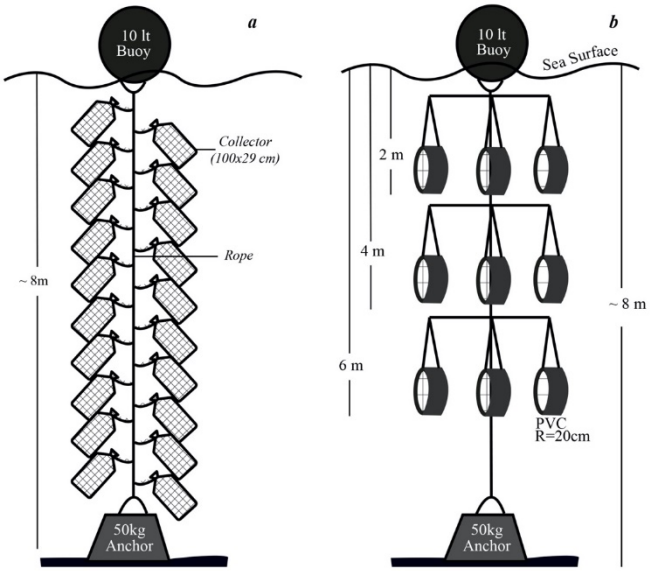

Figure 2. Design of systems: (a) Collector system, (b) Suspended culture system

Environmental parameters

During the study, the water conditions of different depths were monitored and water samples were collected by Niskin water sampler (Model 1010, 5 L). The temperature (°C) was measured monthly with a mercury thermometer, and the temperature of depths was measured with a dive computer (Suunto D4f). The salinity (Practical Salinity Unit - PSU) was analyzed monthly by the Mohr-Knudsen method with water samples taken from different depths (Martin, 1968). The Chlorophyll-*a* and total particulate matter (TPM) values from samples were analyzed according to the method of Strickland & Parsons (1972).

Specific growth rate

The specific growth rates for height (SGRh) and weight (SGRw) were calculated monthly according to the following formulas (Wildish & Saulnier, 1992);

$$SGRh = 100(LnH_t - LnH_0/LnH_0 \times t) \quad (1)$$

H_t is the last height, H_0 is the first height, t is time (30 days).

$$SGRw = 100(LnW_t - LnW_0/LnW_0 \times t) \quad (2)$$

W_t is the final weight, W_0 is the first weight, t is time.

Survival rate

The live individuals were counted in grow-out systems to determine monthly survival. The survival rate was calculated using the equation as below (Lok et al., 2006);

$$Survival\ rate(\%) = 100 - (100 \times \frac{(N_0 - N_t)}{N_0}) \quad (3)$$

N_0 is the number of scallops at the beginning of the experiment and N_t is the number of live scallops at time t.

Statistical Analysis

Descriptive statistics were made using SPSS® statistics program (version 25.0). Kolmogorov-Smirnov test was done for the normality of the distribution of the data. Levene test was done for the homogeneity of variances. Differences between the depths in SGRh and SGRw were determined by a one-way ANOVA test ($p>0.05$). The survival rate was tested by chi square (χ^2). The relationship between water conditions and growth, SGRh and SGRw were determined by the Pearson correlation coefficient. The relationship between survival rate and water conditions was determined by Spearman's correlation test (Zar, 1984).

Results

The highest seawater temperature was measured as 30.1°C at 2 m depth in August, and the lowest seawater temperature was 12.9°C at 6 m depth in February (Figure 3a). Salinity values in the region varied between 36 and 38.19 PSU (Figure 3b). The maximum chlorophyll-*a* value was observed at 8.95 µg l^{-1} in August at 2 m depth (Figure 3c). The maximum and minimum TPM amounts were measured at 10.12 mg l^{-1} and 1.52 mg l^{-1} at 6 m and 2 m, respectively. Average values of TPM amount were calculated as 4.41±1.86 mg l^{-1}, 5.09±1.88 mg l^{-1}, and 5.47±1.89 mg l^{-1} at 2 m, 4 m, and 6 m depth, respectively.

A total of 270 smooth scallops was collected. The average height and weight of smooth scallop spats which were gathered from the collectors were 8.26±1.55 mm and 0.10±0.02 g, respectively.

At the end of the study, the average heights of the scallop spats placed at 2 m, 4 m, and 6 m depth in the suspended culture systems were measured as 42.6±1.11 mm, 41.53±12.85 mm, and 41.57±1.64 mm, respectively. The average weights of the scallops were measured as 12.71±0.89 g, 12.85±0.53 g, and 12.82±1.00 g, respectively (Figure 4).

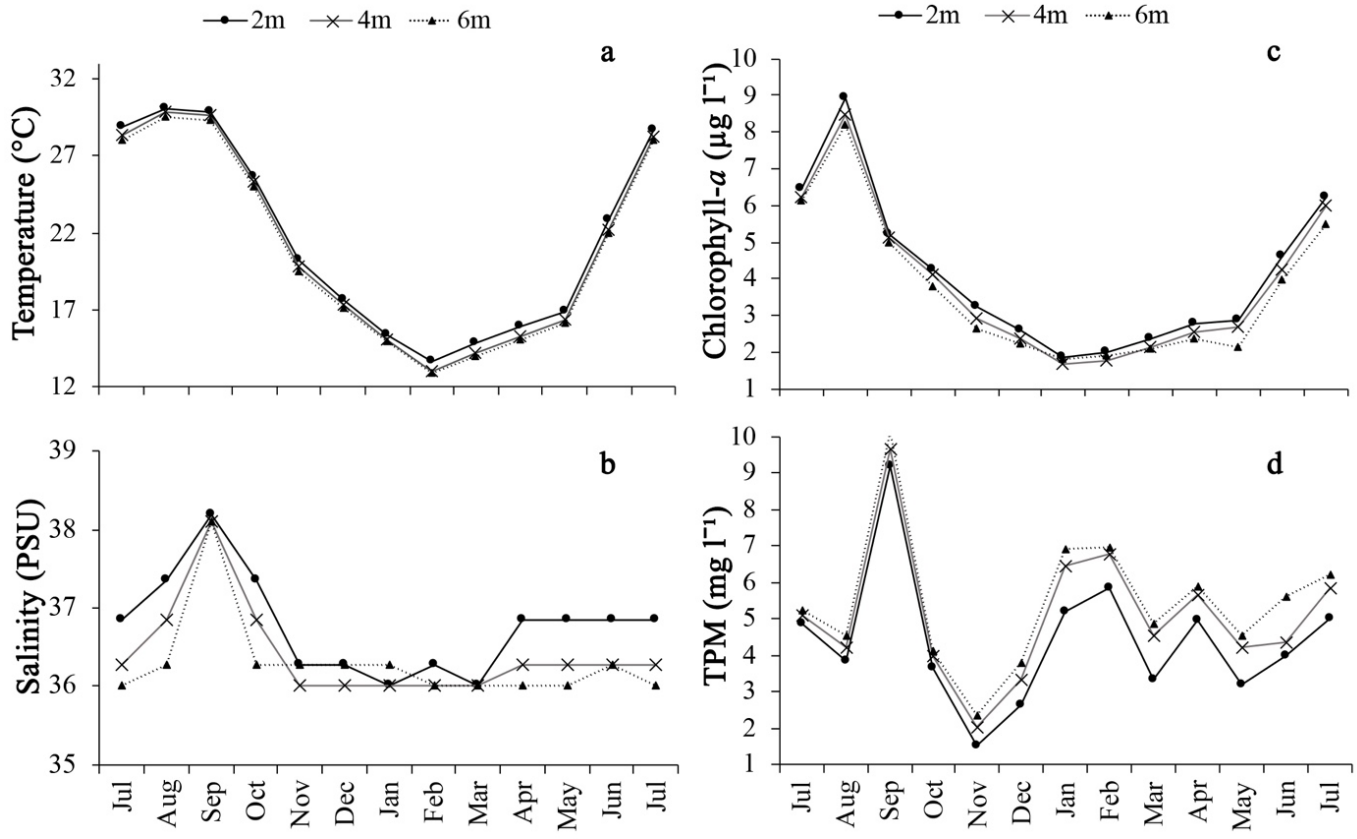

Figure 3. Seawater conditions of the study area (2, 4 and 6 m) (a) Temperature, (b) Salinity, (c) Chlorophyll-*a*, (d) TPM

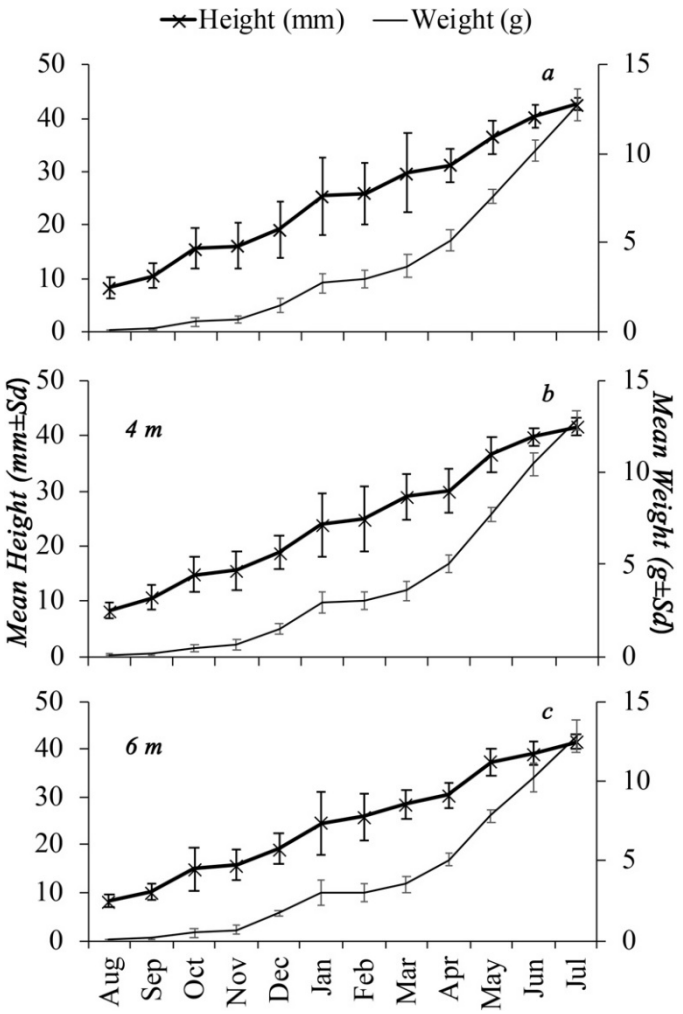

Figure 4. Height -weight change of scallops at different depths (a) 2 m, (b) 4 m, (c) 6 m.

The highest specific growth rate for height and weight of scallops at all depths was observed in October (Figure 5). SGRh at 2 m, 4 m and 6 m depths were determined as 1.62%, 1.33%, and 1.61% and SGRw were found as 9.28%, 7.61% and 6.66% in October, respectively. Daily specific growth rates for height were calculated as 1.156 mm day^{-1} (2 m), 1.112 mm day^{-1} (4 m), and 1.125 mm day^{-1} (6 m) throughout the year. It was determined that there was no statistically difference between specific growth rates depending on height and weight at different depths ($p>0.05$).

The survival rate in scallops was determined as 53.33%, 43.33% and 36.67% at 2 m, 4 m and 6 m, respectively ($p<0.05$) (Figure 6). Significant positive correlations were found between survival rate and temperature parameters with Spearman's correlation for August to February ($r_{2m}=0.961$, $r_{4m}=0.955$, $r_{6m}=0.937$; $p<0.01$).

Discussion

It has been reported that factors such as environmental conditions, predatory organisms, fouling and boring organisms, breeding, and density of individuals in the culture system have affected the growth and survival of bivalves (Yu et al., 2010; Acarlı et al., 2011; Yiğitkurt, 2020). The high growth rate in aquaculture depends on keeping these factors in optimum conditions, especially temperature and salinity are among the environmental factors that largely control the normal development of the scallop (Shumway, 1991; Grecian et al., 2000; Gosling, 2003). In this study, the temperature values varied at all three depths, but the water temperature measured from 2 m depth was higher than other depths. The highest height and weight were measured in individuals at 2 m depth. No correlation was found between specific growth rates and temperature. However, the lowest levels of the size-dependent specific growth rate of scallops were calculated in February at all depths because the lowest temperature was measured in this period. Since scallops are poikilothermic creatures like other bivalves, metabolism and physiological processes generally increase or decrease according to changes in water temperature (Schmidt-Nielsen, 1990). In this study, the water temperature did not reach the lower and upper values that would stop the scallop growth, and it was observed that the growth continued during the periods when the water temperature decreased and increased.

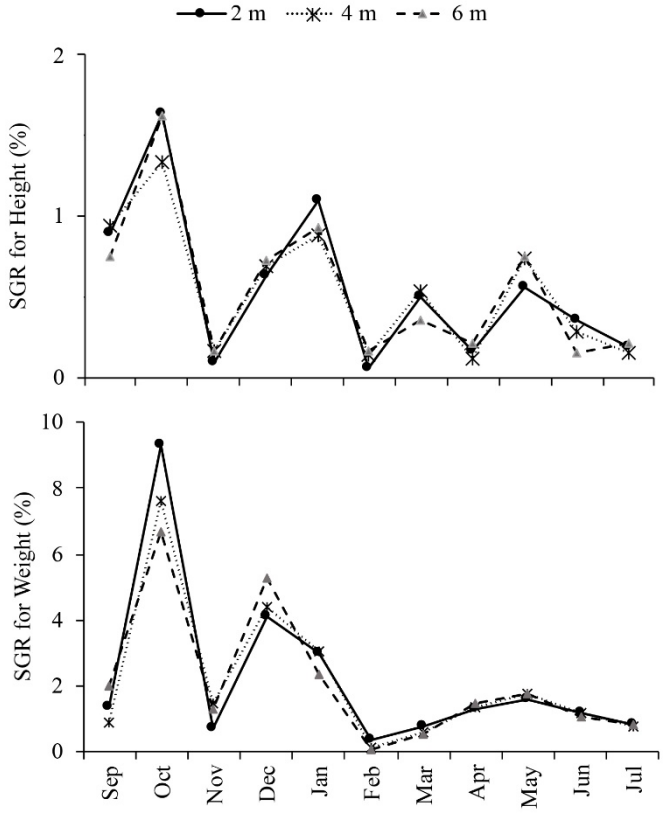

Figure 5. Specific growth rates for height (%) (a) and weight (%) of *Flexopecten glaber*.

The food sources of the scallops are phytoplankton, detritus, bacteria, and other organic substances that it filters from the

water (Bricelj & Shumway, 1991, Beninger & Decottignies 2005). While high nutrient availability is assumed to increase both tissue and gonad growth, nutrient deficiency basically directs metabolic energy to maintain reproductive activity (Delgado & Perez-Camacho, 2005; Yigitkurt, 2021). In this study, seasonal chlorophyll-*a* peaked throughout the water column in July and August. Chlorophyll-*a* started to decrease in September. It was lowest between January and February. No relationship was found between chlorophyll-*a* and SGRh and SGRw. However, SGRh and SGRw values were also lowest in the months when the nutrient level was at its lowest. Chlorophyll-*a* values that were measured in the study area provided the necessary nutrients for scallop growth throughout the year, and nutrient abundance changed in parallel with the increase and decrease in water temperatures, indirectly affecting SGRh and SGRw.

Atmospheric cycles, wave movements, extreme weather changes and high rainfall in shallow sea areas significantly affect TPM levels (Orpin et al., 2004). Szostek et al. (2013) reported that resuspension of sediments would affect the growth, feeding and survival rates of scallops. There was little difference between concentrations at the depths. The seasonal variation in TPM was very erratic. Peaks occurred in September. The lowest TPM values at all depths were determined in November. SGRh and SGRw values were at low levels in November, but no correlation was found between TPM and SGRh and SGRw values.

Louro et al. (2005) stated that the scallops *Pecten maximus* that are 3 mm placed in suspended culture systems, reached 17 mm at the end of 85 days, the spats placed in the system as 4 mm size, grew up to more than 17 mm at the end of 57 days. At the same time, the survival rate was 70%. In a study in Ria de Arosa (Galicia, Northwest Spain) that lasted 259 days (October-July), it was reported that *Aequipecten opercularis*, one of the scallop species, increased from 22 mm to 58 mm with a specific growth rate of 0.11 mm day^{-1} (Roman et al., 1999). In a study conducted in the North Adriatic Sea, it was reported that *F. glaber*, which was placed in the system with a size of approximately 18 mm during the culture period, reached 35 mm height at the end of 390 days with a specific growth rate of 0.08 mm day^{-1} (Marčeta et al., 2016). In this study, the 8 mm *F. glaber* spats were grown at different depths for 12 months (360 days), and the best growth was found as 42.6±1.11 mm at a depth of 2 m with a daily specific growth rate of 1.156 mm day^{-1}. Comparing the findings of this study with other studies, the daily specific growth rate is higher in the present study. There was no statistically significant difference in specific growth rates between depths ($p>0.05$), but there was a statistically significant difference between the specific growth rate by months ($p<0.05$). Due to the small size of the individuals used in the experiment, we encountered high specific growth rates at the beginning of the study, and these rates continued to decrease in the following months of the study.

Causes such as wave motion, contamination, nutrient limitation, stock density have been reported as factors that increase the mortality rate (Grecian et al., 2000). In addition, Duggan (1973) reported that deaths increased as the shells of the scallops cut the soft tissue of the other scallop with the wave movements in the culture systems and called this "stabbing". It has been determined that the increase in mortality rates and the increase in the height of the scallops in the growing systems and

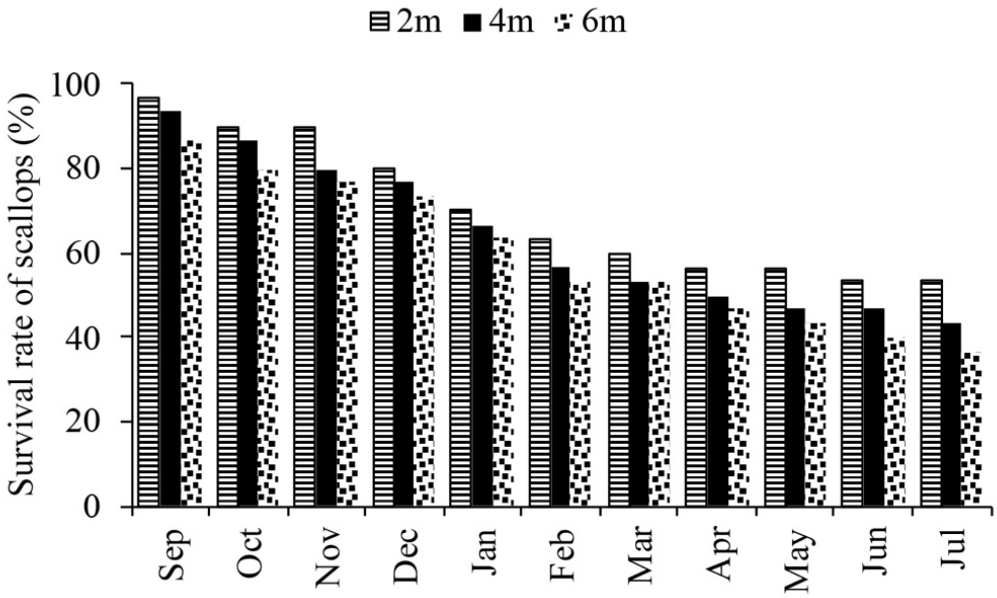

Figure 6. Survival rates of *Flexopecten glaber* (%) at different depths (2 m, 4 m and 6 m)

the narrowing of the area they are in cause harm to each other. At the end of this study, the mortality rates at 2 m, 4 m and 6 m depths were determined as 46.67%, 56.67%, and 63.33%, respectively. A strong positive correlation was found between mortality rate and height growth (r_{2m}=0.941, r_{4m}=0.956, r_{6m}=0.982). In culture systems, as the individual height increased, the mesh size was changed, but the culture system dimensions were kept constant, which increased the mortality rate. It has been reported that growth slows down, and high mortality rates are seen in growth systems near the bottom due to high TPM concentrations (Duggan, 1973; Emerson et al., 1994). This study also observed that the mortality rate increased in growth systems close to the bottom, and TPM rates were higher at 6m depth, which can be explained by the high TPM values.

Conclusion

In conclusion, the growth of scallops in suspended culture systems has been carried out for the first time in the region. In this study, the culture of *F. glaber*, one of the scallops known as potential species for culture in İzmir Urla Karantina Island and its surroundings, has been deemed appropriate due to the survival rate of approximately 50%. Even so, determining new potential areas for culture operations of this species is important in terms of higher survival rate and growth performance. Thus, this species' culture will contribute to the country's economy and create a new employment area and a new alternative product for export. For this species, it is recommended to investigate sexual maturity times and sizes in future studies.

Compliance With Ethical Standards

Conflict of Interest

The author declares that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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Marine Science and Technology Bulletin

REVIEW PAPER

Current insights on wastewater treatment and application of *Spirulina platensis* in improving the water quality

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ABSTRACT

Excessive generation of wastewater is one of the major reasons for pollution in natural reservoirs. Given the normal circumstances, natural water bodies revive and rejuvenate themselves; but upon increased waste load, the self-revival system of the ecosystem slows down, causing water pollution. Hazardous waste, especially heavy metals and organic pollutants, have affected the ecology to the detriment of humans. Thus, the need arises for wastewater treatment, before its discharge. Current methods undertaken include the use of physical settling of solid waste, filtration, aerobic and anaerobic microbes, and chemical treatments. Low removal of pathogens, dependence on the uninterrupted power supply, high maintenance cost, generation of explosive biogas and bioaccumulation of chemicals are some disadvantages of activated sludge technology, one of the modern technologies used. Hence, the focus has been shifted on organisms capable of metabolizing, immobilizing or absorbing toxic compounds from their environment, making it both environment-friendly and cost-effective. This review provides perspicacity about the generation of sewage and the various methods available for its treatment. Emphasis is made on bioremediation using *Spirulina platensis*. Since the organism assimilates the bioavailable contaminants of sewage water photosynthetically; it can overcome the demerits of conventional methods. It also discusses possibilities of using *Spirulina* grown on the sewage as a food supplement, animal fodder or source of bioactive compounds.

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Introduction

Anthropogenic pressures on the environment bring about radical transformations in the ecosystem; leading to imbalance and long-term effects. Water pollution causes alterations in water quality and negatively affects the proper use of water. One of the major social and economic concerns is the scarcity of potable water. Thus, it has become a need of the hour to secure this natural resource.

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The present domestic wastewater collection, treatment and disposal amenities, in Mumbai, were given in the 1880s by the governing civic body - Brihanmumbai Municipal Corporation (BMC) - and continued with some development (Gupta et al., 1998). Around 2700 million litres per day (mld) water out of the 3700 mld that is provided by the municipality is tossed into the rivers, lakes, streams or oceans untreated, which accounts for 90% of total wastewater discharged (UNEP Document Repository, 2002). With the waste overload, the self-revival and self-purification systems are disrupted, leading to problems such as excessive utilisation of dissolved oxygen (DO) (Sperling, 2007).

Developed countries or regions have been dealing with the problem of water pollution by removal of nutrients and micro-pollutants especially biodegradable organic matter and pathogens, together with attention to stormwater drainage (Amin et al., 2014). Treatment of wastewater is of utmost importance to be able to recirculate the finite resource of water on Earth.

Heavy metal contamination is a global concern as improper discharge poses serious threats to the environment and public health due to persistence, biomagnification and bioaccumulation (Rehman et al., 2007; Wang & Chen, 2009; Samantaray et al., 2014; Beauvais-Flück et al., 2018). Heavy metals cannot be biodegraded but can be biotransformed (Boopathy, 2000; Juwarkar et al., 2001; Wexler, 2004). Their bioavailability and toxic potential influence the pH, organic matter and nutrient status of water. Consequently, heavy metals may be potentially toxic and hazardous to plants, animals and ultimately humans (Tchounwou et al., 2012).

The focus is on sustainable development for a sustainable environment, due to health-related issues of environmental pollution (Elleuch et al., 2018). Balancing the thin edge between the advancement of technological and economic development, and environmental conservation has been the main focus of sustainability and sustainable development. Human health and examining the long-term outcomes of the actions are aspects that sustainability deals with, apart from just about the environment (WCED, 1987; Boopathy, 2000).

Bioremediation

Bioremediation or Green Remediation involves the biological deterioration of contaminants into less toxic or non-toxic compounds, using plants, fungi and/or microorganisms (Pierzynski et al., 1994; Abou-Shanab et al., 2011). It is a tactic of taking into account all ecological effects of remedy implementation and incorporating options to ratchet up net environmental benefits of cleanup actions. It aids in mineralizing organic pollutants, partially transforming them or altering their mobility. Bioremediation intensifies the natural rate of degradation of contaminants by facilitating the indigenous microbes, fungi or plants with nutrients, carbon sources, or negatron donors (biostimulation, bio-restoration) or by supplementing enhanced microbial cultures that have distinct characteristics to degrade the required pollutant at a faster rate (bioaugmentation) (Mackay & Frasar, 2000; Gouma et al., 2014).

Bioremedial agents use organic carbon from the contaminants as an energy source, in turn breaking down the contaminants (Boone & Castenholz, 2001). Some bioremedial organisms might not mineralize the pollutant instead produce a more potentially toxic compound (Wexler, 2004), but the use of a different organism can work efficiently. Thus, it is important to understand the metabolic and chemical pathways of the said organism, before its use in bioremediation (Juwarkar et al., 2001).

Spirulina platensis

Spirulina Turpin ex Gomont, 1892 was first isolated by P. J. Turpin from a freshwater lake. *Spirulina platensis* (Gomont) Geitler 1925 (Figure 1) is a non-branched, helicoidal, filamentous, Gram-negative cyanobacteria (Whitton, 1992; Boone & Castenholz, 2001; Sánchez et al., 2003). It is identifiable by the organisation of the multicellular barrel-shaped trichomes in an open left-hand helix on the entire length. They show a transverse cross wall and seem as a non-heterocystous filament (Paramanya et al., 2019); these filaments are composed of vegetative cells that experience binary fission in a single plane (Vonshak et al., 1996; Habib et al., 2008). Its cell wall comprises a weak envelope, created of multiple layers of peptidoglycan and lipopolysaccharide (Wan et al., 2016). Spirulina is the commercial name for *Spirulina platensis*, from which a blue pigment - phycocyanin - is extracted (Eriksen, 2008).

Figure 1. External morphology of *Spirulina platensis* (Koristka, 1891)

The major photosynthetic pigment in *Spirulina* sp. is phycocyanin; it conjointly includes chlorophyll a and carotenoids with it. Some may also contain phycoerythrin, a reddish pigment; these pigments facilitate *Spirulina* sp. to absorb energy from the sun (Habib et al., 2008; Capelli &

Cysewski, 2010; Mary Leema et al., 2010). It is an obligate photoautotroph (Barrón et al., 2007); in light conditions, it reduces carbon dioxide and chiefly assimilates nitrates (Richmond, 1986). They also have a mutualistic interaction with nitrogen-fixing bacteria that fix nitrogen from the air (Vo et al., 2008).

It thrives naturally in alkali soda lakes, especially in Africa and Mexico (Ciferri, 2008; Wan et al., 2016). Saline water (>30 g/L) with pH 8.5-11.0 favours good growth, especially in areas with higher sun's radiation (Vo et al., 2008); Optimum growth is between 35-39°C (Richmond, 1986). Extensive Cultivation of *Spirulina* sp. is straightforward and ensures high biomass for extraction, separation and refinement of high-value bioactive compounds (Barrón et al., 2007). Microalgae like *Spirulina* sp. are 'biofactories' that have rapid growth and low nutrient necessities (Masojídek & Torzillo, 2008). Contamination is low as many do not flourish in the high salt environment and generally grow as a unialgal population (Komárek & Lund, 1998).

Spirulina sp. consists of 55-70% protein, 15-25% carbohydrate, 5-6% total lipids, 6-13% nucleic acids (DNA and RNA), and 2.2-4.8% minerals; 25% branched carbohydrate of the total polysaccharide is structurally comparable to glycogen (Hosseini *et al*., 2013). It constitutes 1.5-2% Polyunsaturated Fatty Acids (PUFAs) of the total lipid content (Wan et al., 2016). Different non-saponifiable lipids such as paraffin, terpene alcohol and sterols are present (Falquet, 2008). It is a source of vitamins, viz. B1 (Thiamine), B2 (Riboflavin), B3 (Niacin), B6 (Pyridoxine), B9 (Folate), B12 (Cobalamin) and minerals (Cu, Ca, Fe, Cr, Mn, Mg, P, Se, Na, and Zn).

Since the 1500s, it has been a popular food supplement for diets of humans, poultry, livestock and aquaculture – a comprehensive protein source (Wan et al., 2016; Paramanya et al., 2019). Most blue-green algae produce toxins, microcystins, but food supplements of *S. platensis* have so far been cleared for consumption (Koristka, 1891; Chamorro & Salazar, 1990). If accurately defined free from contaminants and adulterants, it is considered 'Generally Recognized as Safe (GRAS)' by the Food and Drug Administration (Paramanya et al., 2019).

Given all these nutritional qualities of the microalgae, its use in long term use as dietary supplements in Mars missions and other space flights is being scrutinized for (Paramanya et al., 2019). Also, its use in developing countries for malnutrition was suggested by the World Health Organization (WHO) in 2012. The potential applications in combating the food crisis bring forth the necessity for effective and mass production of *Spirulina* sp. to achieve the requirement.

Phycocyanin is used as a natural colouring agent in food and beverages (Buchweitz, 2016). It has pharmacological activity and a clinically therapeutic effect (Paramanya et al., 2019). Other bioactive compounds in *Spirulina* sp. are γ-linoleic acid (Wan et al., 2016; Tanticharoen et al., 2009), sulfated polysaccharides (Hayashi et al., 1996), sulfolipids (Barrón et al., 2007), and insulin-like protein (Anwer et al., 2012). *Spirulina* sp., other than its popular and extensive application in the food industry and pharmaceuticals, in recent times, is used in genomic studies and also as a model for physiological studies, especially to understand nitrogen assimilation. *Spirulina* sp. has also found an application in bioremediation.

Spirulina sp. in Bioremediation

The environment is susceptible to heavy metals because of their persistence and toxicity (Samantaray et al., 2014). Many industries discharge untreated heavy metal waste and pollute soil or water resources. These non-biodegradable pollutants accumulate in the food web (bioaccumulation) and disturb the ecological balance by magnifying up the trophic levels (biomagnification). Conventional water treatment is dominated by physical and chemical strategies, and traditional infrastructure, represented by classical control and treatment plants, viz., evaporation, ion exchange, electroplating, membrane processes or precipitation, to eliminate persistent heavy metals, particularly from liquid waste. They are also high in energy consumption and quite expensive (Priyadarshani et al., 2011).

The enormous potential of biotechnologies in bioremediation is unjustifiably ignored (Cepoia & Zinicovscaia, 2002). Bioremediation can complete and enhance the efficiency of traditional technologies. Microorganisms have evolved many bioprocesses in the presence of heavy metals, viz. Pb, Cu, Cd and Zn from wastewater below 50 mg/L, such as transportation across the cytomembrane, biological adsorption to outer cell walls and accumulation in extracellular capsules, precipitation and redox reactions (Chen & Pan, 2002). Microbes exploit the chemical properties of the metals that are selectively acquired, for catalyzing reactions and also for maintaining protein structure (Murali & Mehar, 2014).

Due to their ability to survive extreme environments and metabolise a variety of compounds, cyanobacteria are promising in degrading peculiar pollutants and removing heavy metals from wastewater (Kumar et al., 2016). It was found that cyanobacterial growth depends solely on the penetrating light and temperature, whereas nutrients and agitation are not the limitations (Torzilla & Vonshak, 1994). Various systems, therefore, are tested for their applicability to enhance the effectiveness and economic feasibility (Martinez et al., 2000). *Anabaena variabilis, Lyngbya majuscule, Nostoc muscorum* and *Oscillatoria salinas* are established

cyanobacterial treatment systems, especially for textile industry effluents (Kumar et al., 2016).

Cyanobacteria perform photosynthesis in distinctive folds instead of chloroplasts, as they lack internal membrane-bound organelles like green plants. Several functional groups (carboxyl, phosphonate, amine and hydroxyl groups) are present on the bacterial cell wall that helps with metal binding and biosorption (Doyle et al., 1980). Certain cyanobacterial strains produce Extracellular Polymeric Substances (EPS), which potentially absorb heavy metals from their surroundings (Kulkarni et al., 2010). Biosorption on lignocellulosic wastes and by-products was identified as an alternative to the existing waste management technologies applied for toxic metal ions and dyes. Studies have demonstrated the use of bacteria as biosorbents - for example, *Streptomyces* sp. (Saurav & Kannabiran, 2011). Since marine environments are characterised by the presence of high salt and ion contents, the use of marine organisms for bioremediation of metals present in sewage is considered (Deng & Wang, 2012).

Spirulina sp. can be cultivated worldwide, in hot and alkaline environments; the culture ensures hygiene, as no other organisms can survive and contaminate the culture. There is very little information about the use of *Spirulina platensis* in bioremediation technologies (Soeprobowati & Hariyati, 2014) but has been considered an excellent candidate for bioremediation due to its tolerance for toxic heavy metals and biosorption capacity (Murali & Mehar, 2014; Çelekli et al., 2012). It was first used for domestic wastewater treatment in 1974 (Dolatabadi & Hosseini, 2016). The bioremediation potential of *Spirulina* sp. has also been utilised to exclude heavy metals and limit the saturation of nitrate and phosphorus in water bodies (Fariduddin et al., 2017).

Additionally, this cyanobacterium is also used for bioremediation of water polluted with petroleum hydrocarbons (Ciferri, 1983), pesticides (Khan et al., 2005), estrogens (Shi et al., 2010), radioactive elements (Fukuda et al., 2014) and fluoride ions (Tabagari et al., 2019). *Spirulina* sp. was used for biosorption of Cr^{3+}, Cd^{2+} and Cu^{2+} ions (Chojnacka et al., 2005).

Culturing *S. platensis* in low metal concentrations can be potentially used for tertiary treatment of metal-contaminated effluent (Dolatabadi & Hosseini, 2016). Several researchers have also attempted to find suitable desorbing solutions to recover metals from the biomass after cultivating it on wastewater; such reuse of the biological sorbent is better at addressing both environmental and economic issues (Mehta et al., 2002). Miazek et al. (2015) suggested the use of metal-containing wastewater as replenishment for microalgae growth in nutrient-deficient media.

Spirulina sp., though has a meagre capacity to bind to metal ions and a low tolerance for heavy metals, has considerable potential to precipitate them (Fariduddin et al., 2017). Using *Spirulina* sp. as a precipitation agent has gained attention recently; the passive metal ion adsorption rate was observed to be accelerated in *S. platensis* (Doshi et al., 2007). A study by Kőnig-Péter et al. (2015), studied the biosorption efficiency of *S. platensis–S. maxima* cells in a system containing 1.0 g/l; it was found that about 80% of the heavy metal ions could be removed, in the optimal pH range of 4-6. The specific adsorption of both Pb^{2+} and Zn^{2+} increased at low concentration and decreased when dead *S. platensis* biomass concentration exceeded 0.1 g/l (Palaniswamy & Veluchamy, 2017). A study by Al-Homaidan et al. (2015) showed greater than 91% of Pb^{2+} removal at a concentration of 2g of *S. platensis* in 100 mg/l of lead initial concentration (pH 3; 26°C). *S. platensis* has great potential as an eco-friendly bio-adsorbent for the removal of Copper from aqueous solutions (Çelekli et al., 2012). *S. platensis* has shown to be effective in bioremediating dyes; the potential was enhanced using micro or nanoparticles (Dotto et al., 2019). Thereby, it can assist in designing and developing low-cost approaches for the large-scale synthesis of nanoparticles and bioremediation approaches (Priyadarshini et al., 2019).

Nitrate and phosphorus contamination is another major problem of freshwater sources. Due to its mixotrophic nature, *S. platensis* has the potential to reduce biological oxygen demand (BOD) of high carbon-containing wastewater (Kulkarni et al., 2010). Application of *Spirulina* sp. to minimise and eliminate nitrate and phosphate from the wastewater is established (Fariduddin et al., 2017).

Most of the research conducted for the exclusion of heavy metal from wastewater used dead biomass. The use of dead biomass is favourable - it can tolerate high concentrations of toxic heavy metal ions, nutrient supply is not unnecessary and culture conditions are not limiting (Mehta et al., 2002; Kőnig-Péter et al., 2014); thus, a potentially cost-effective way of removing toxins from industrial wastewaters (Rangsayatorn et al., 2004). Studies about processes for heavy metal uptake by dead biomass *Spirulina* are available (Rangsayatorn et al., 2004), but sparsely for the use of its live biomass. Doshi et al. (2007) first proposed live *Spirulina* sp. as a stronger candidate than dry biomass for the management of industrial wastewater. Future work is necessary as many uncertainties are associated with the development of wastewater treatment by live biomass of *Spirulina* sp. (Chen & Pan, 2002).

Chen & Pan (2004) concluded that living *Spirulina* cells had a high tolerance to lead (concentration below 50 mg/L) and are good adsorbing agents. The growth response of *Spirulina* to

toxic heavy metals depends on the metal involved as in its mechanism of tolerance (Thomson & Kurup, 2010). According to Thomson & Kurup (2010), Zinc (up to 10 µg/ml) and Iron (up to 100 µg/ml) showed a growth-promoting effect in *S. platensis* and established the order of toxicity, Hg>Ni>Cu>Zn>Fe (Thomson & Kurup, 2010). Murthy et al. (1989) demonstrated that Mercury ions at low concentrations affect the transfer of energy within phycobilisomes and at high concentrations showed 50% inhibition of Hill activity in *S. platensis* (Murthy et al., 1989). In another experiment, heavy metal-treated *S. platensis* showed capability for the accumulation of tenfold more Cu and Zn than control cells (Nalimova et al., 2005).

No concrete evidence for the mechanisms of heavy metal toxicity in *S. platensis* has been reported (Nalimova et al., 2005), but it can be related to the precipitation of heavy metals, reduction to unavailable compounds or production of complexes with secreted metabolites Tomsett & Thurman (1988).

Use of Sewage as the Medium for S. platensis and its Applications

In India, National Environmental Engineering Research Institute (NEERI), Nagpur has developed an algae cultivation technique in sewage oxidation pond systems. While, mass production of Single Cell Protein (SCP) from cyanobacteria on sewage is established by National Botanical Research Institute (NBRI), Lucknow and Central Food Technological Research Institute (CFTRI), Mysore. The SCP is produced and is further utilised as animal feed (Kulkarni et al., 2010). Algae on sewage serve a dual purpose of cleaning up potential environmental pollution and producing valuable protein. Similarly, an effective and cost-efficient technique needs to be established for cyanobacteria.

Wastewater is used as a source of nutrients; *S. platensis* is found to grow better in diluted sewage when improved with sodium carbonate or sodium bicarbonate and nutrients in different proportions. This further screened, is added to aquaculture to feed fish or dried in a small solar drier for animal feed (Kulkarni et al., 2010). Soeprobowati & Hariyati (2014) suggested the use of *S. platensis* as a metal absorbent and further as fertiliser, after bioremedial processes. Lead, Mercury, Cadmium and Arsenic are common in agricultural areas and also frequently adulterate *Spirulina* cultures (Al-Dhabi, 2013).

Michalak et al. (2019) studied the potential of *S. platensis* as a biosorbent for nutrients. They further also proposed its application as a valuable carrier of metal ions for the production of bioactive additives for the improvement of insulin resistance in horses. The commercially grown and consumed *Spirulina* supplements have traces of inorganic elements and heavy metals, in the concentration that do not exceed the present regulation levels. If appropriate measures are taken, they can be considered safe food (Al-Dhabi, 2013). This application is the potential to combat the issues of contaminated biomass.

Some changes in the *Spirulina* sp. occur after being used to bioremediate sewage. Studies report a decrease in the biomass of *Spirulina platensis*, pigment (phycocyanin) concentration and changes in their chemical pathways when grown on metal-rich medium or sewage (Doke et al., 2015). The rising concentration of heavy metals undeviatingly affects the phycocyanin generation in *Spirulina* sp. (Doke et al., 2015). Cu and Zn were reported to directly affect the photosynthetic pathways, resulting in a decline in cell growth (Nalimova et al., 2005). In vivo, Mercury results in the breakdown of both light and dark photosynthetic reactions, by substituting the central atom of chlorophyll, magnesium (Patra & Sharma, 2000).

Changes and variations in the biomass yield and chemical composition of *Spirulina* should be considered for extracting bioactive compounds or in its application as a food supplement or fertiliser. *Spirulina platensis* is a potential organism for a cost-effective and environment-friendly wastewater treatment technique.

Conclusion

Overpopulation and heavy industrialization in the 2000s have increased the generation of wastes and sewage, which when treated is let out in the water bodies. Physicochemical methods of sewage treatment are mostly environmentally degrading and require heavy machinery, increasing the cost of the process. Consequently, the centre of attention has shifted to bioremediation. Microorganisms utilise the organic and inorganic nutrients from the sewage, thereby reducing the pollutant load. Photosynthetic cyanobacteria, such as *Spirulina platensis*, can thrive and reproduce in lower bioavailability. Because of their simple growth requirements such as sunlight, carbon dioxide; easiness in their genome manipulations and well-documented applications, there is a need and a good chance for optimal utilisation of cyanobacteria. Hence, *Spirulina platensis* is an organism of choice for bioremediation.

With several bioactive compounds and catalytic enzymes, this cyanobacteria has proven to degrade heavy metals as well as biological contaminants. Moreover, the biomass generated during sewage treatment can be utilised to extract commercially valued products (bioactive compounds) or nutrient-enriched food supplements for humans and animals.

Even though *S. platensis* has many advantages, it needs to be explored for using its potential at maximum levels. Its industrial application for mass production of the desired compound is technologically challenging and will need high-efficiency photosynthetic bioreactors with minimum operating costs. There is a need to develop cultivation systems that harness the photosynthetic capability of *S. platensis* for discovering green paths to produce industrial products. That being said, *S. platensis* is a potential, dual solution for combating issues of wastewater treatment and mass-production for further application.

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Compliance With Ethical Standards

Authors' Contributions:

Author AP1 and AA designed the study, AP1, AP2 and PP wrote the first draft of the manuscript, AP2 corrected and formatted the manuscript, AA reviewed and finalised the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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RESEARCH ARTICLE

Impact of nutrient load coming from Göksu River on the northeastern Mediterranean

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ABSTRACT

Even though North-Eastern Mediterranean (NE Med) is classified as oligotrophic, inshore areas are highly eutrophic due to the discharge of silicate and nitrate-rich surface waters. Aim of this study was to investigate the nutrient load coming from the Göksu River and to estimate its impact on the river domain using satellite images. Monthly, average nitrite (NO_2), nitrate (NO_3), ammonium (NH_4) and phosphate (PO_4) load found to be varying between 0.07-31.2 ton/month, 15-1226 ton/month, 0.5-539 ton/month, 1-267 ton/month, respectively. Satellite images showed that surface chlorophyll–*a* (chl-*a*) in the river downstream had an increase in both winter and spring seasons as a result of intense precipitation; while, primary production at the offshore regions was mainly impacted by winter mixing and summer stratification. The highest chl-*a* concentration was observed at the river impacted zone and decreased by more than two folds at the offshore regions. Increased NO_3 load observed during winter and spring leads to phytoplankton blooms in the river downstream. The high P content of Göksu River surface waters has increased the productivity at all seasons. As a consequence, correlation analysis showed significant relationship between surface chl-*a* concentration and PO_4-NO_3 load.

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Introduction

Human-induced activities cause variations in the land use patterns that lead to the alteration in both quality and quantity of surface runoff reaching the surface waters. Surface waters become vulnerable for several types of polluters including high nitrogen and phosphorus concentrations (Song, 2009). Incoming pollutant and nutrient load cause an alteration in the bio-physicochemical balance of receiving environments (Kangur & Möls, 2008) such as decrease in dissolved oxygen concentration, light penetration. This phenomena is called as eutrophication (Nixon, 1995).

The inshore area of Northeastern Mediterranean (NE Med) is classified as eutrophic due to the discharge of silicate and nitrate-rich surface waters (Tugrul et al., 2016); whereas, the offshore area is classified as oligotrophic (Krom et al., 1991).

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Göksu River; among other surface waters; is mainly suffered from domestic and agricultural discharges that increase the organic content of the surface flow. These nutrient-rich waters could not diffuse offshore due to the blockage of Asian Minor Current (AMC) which increases the eutrophication risk of the receiving environment (Akpınar et al., 2016).

Knowledge of nutrient load coming from surface waters to open sea is crucial; since it directly affects the chemical and biological loop in the surrounding environment. This study was conducted to (i) determine the nutrient load coming from Göksu River (NH_4, PO_4, NO_2, and NO_3), (ii) to evaluate the seasonal variations in the nutrient load, (iii) to determine the impact of Göksu River on the primary production using satellite images.

Material and Methods

Göksu River descends from the Taurus Mountains and discharges its waters to NE Med from Silifke. As a result of the Mediterranean climate, its flowrate starts to increase during winter, and reach its maximum level in spring due to the rainy season, and decrease in the hot summer as a consequence of the powerful evaporation (DSİ, 2019). Almost 95% of the Göksu Basin is forest, semi-natural areas, and agricultural lands (Figure 1). So, the Göksu River carries a significant amount of nutrient load to the NE Med (Ayaz et al., 2013).

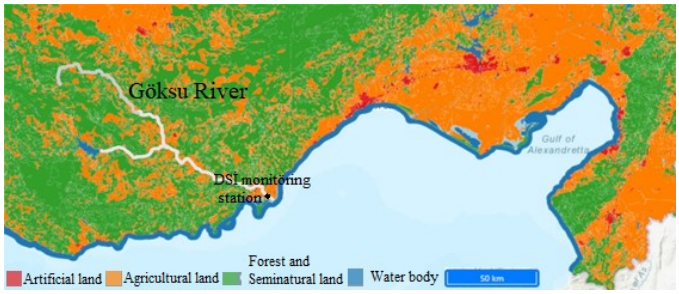

Figure 1. Land use map of Göksu River surrounding

Monitoring study results that belong to the downstream of Göksu River station were taken from the State of Hydraulic of Turkey including seasonal flow rate (Q), nitrite (NO_2), nitrate (NO_3), ammonium (NH_4), and phosphate (PO_4) concentrations from 1992 to 2016. Dataset was used to calculate nutrient load using an interpolation-based average estimator model (Quilbé et al., 2006) (Eq. 1).

$$Load = K\left(\sum_{i=1}^{n}\right)\left(\frac{C_i Q_i}{n}\right) \quad (1)$$

where;

K = conversion factor to take account of the period of record

C_i = instantaneous concentration associated with individual samples (mg/L)

Q_i = instantaneous discharge at the time of sampling (m^3/L)

n = number of samples

Satellite images were used to evaluate the impact of Göksu River on NE Med by evaluating the surface chlorophyll-*a* distribution. Satellite images were obtained from NASA's 3rd level browser belonging to the Ocean Color Web application (https://oceancolor.gsfc.nasa.gov/). NASA emerges in-situ chl-*a* measurements with remote sensing to predict the surface chl-*a* concentration. Current implementation, algorithm description and constraints of the model was described in Morel & Maritorena (2001), Hu et al. (2012) and can be obtained from official webpage (https://oceancolor.gsfc.nasa.gov/atbd/chlor_a/). The monthly chl-*a* images were downloaded from the MODIS-Aqua sensor with a resolution of 4 km (NASA Goddard Space Flight Center, 2014) and processed using the SeaDAS program (Baith et al., 2001). Surface chl-*a* imaging was coherently conducted to the nutrient sampling time.

Pearson correlation analysis was used to test the existence of a linear relationship between the surface chl-*a* concentration obtained from satellite images and nutrient load.

Results and Discussion

Properties of Göksu River Surface Waters

Coastal area of NE Med is fed by nutrients coming from surface waters (Tugrul et al., 2016) which could not be penetrate to the offshore due to the blockage of AMC (Akpınar et al., 2016). For that reason, eutrophic conditions prevail in coastal areas, which make monitoring of upcoming nutrient load from surface waters an issue. As a result of these concerns, several studies were undertaken in the Göksu River basin. Demirel et al. (2011) reported that the NO_2, NO_3 and PO_4 concentration in different parts of the Göksu basin as 0.03-1.31 mg/L, 3.6-17.3 mg/L, and 0.03-0.88 mg/L, respectively. Yıldırım et al. (2018) reported that the NO_2 concentration in the Göksu River ranged between 0.001-0.091 mg/L in October, reaching to 0.107-1.46 mg/L in May. They also reported the NO_3 concentration variation as 2.69-7.95 mg/L and 1.77-5.9 mg/L in October and May, respectively. Similar to the previous studies, in this study, NO_2, NO_3, NH_4 and PO_4 concentration were varied from 0.001 mg/L to 0.114 mg/L, from 0.18 mg/L to 4.8 mg/L, 0.008 mg/L to 2.8 mg/L and from 0.01 mg/L to 0.92 mg/L, respectively (Table 1).

Table 1. Nutrient concentration in the downstream of Göksu River and estimated nutrient load

Year	Concentration (mg/L)				Load (ton/year)			
	NO_2	NO_3	NH_4	PO_4	NO_2	NO_3	NH_4	PO_4
1992	0.002	0.84	0.33	0.03	17	4 264	850	260
1993	0.003	0.71	0.26	0.08	17	2 720	1 831	215
1994	0.006	0.62	0.27	0.10	8	1 147	314	107
1995	0.003	0.89	0.47	0.06	3	1 150	593	66
1996	0.003	0.59	0.22	0.11	13	2 225	948	444
1997	0.005	0.79	0.24	0.11	19	2 476	956	368
1998	0.007	0.83	0.19	0.24	19	2 502	403	863
1999	0.006	0.86	0.15	0.11	17	2 875	399	252
2000	0.009	0.95	0.43	0.12	36	3 418	747	418
2001	0.009	0.81	0.57	0.09	44	3 071	2 472	362
2002	0.013	0.77	0.62	0.12	28	2 293	1 393	300
2003	0.005	0.66	0.53	0.10	12	1 646	1 256	268
2004	0.008	0.80	0.46	0.15	17	1 912	1 032	436
2005	0.010	0.87	1.06	0.15	15	1 431	1 756	264
2006	0.014	0.85	0.64	0.13	18	1 526	1 021	219
2007	0.010	0.97	1.54	0.09	9	1 046	1 578	94
2008	0.004	1.02	0.36	0.08	4	1 172	490	90
2009	0.004	0.59	0.09	0.06	13	1 665	236	123
2010	0.016	0.85	0.25	0.41	15	1 209	246	955
2011	0.018	1.15	0.31	0.28	22	1 645	525	546
2012	0.039	1.36	0.42	0.38	66	2 466	1 060	479
2013	0.056	2.15	0.38	0.14	196	7 304	1 338	486
2014	0.058	1.25	0.25	0.11	105	1 819	547	167
2015*	0.033	1.20	0.25	0.01				
2016	0.017	1.45	0.06	0.23	48	2 585	158	649
2017	0.041	0.81	0.02	0.06	74	1 789	37	110
Mean	0.015	0.95	0.40	0.14	33	2 294	887	342

Note: *load calculation could not be carried out due to missing instantaneous flowrate data.

According to the quality standards of Surface Water Quality Management Regulation (Ministry of Forestry and Water Management, 2015) which determines the procedures and principles required to protect the water quality of surface waters in Turkey, Göksu River was suffered from nitrite, ammonium, and phosphate pollution (Figure 2). Depending on this high nutrient load, Göksu River and its downstream were categorized as sensitive area (Ayaz et al., 2013; Ministry of Forestry and Water Management, 2016).

Nitrite and nitrate salts are chemically active in water (Birkinshaw & Ewen, 2000; Shamrukh et al., 2001; Demirel et al., 2011), and their presence in water usually associated with agricultural activities (Ledoux et al., 2007; Ogwueleka, 2015). Similarly, the presence of dissolved phosphate in water is usually linked with fertilizer usage in agricultural activities (Sing et al., 2005; Ogwueleka, 2015), as well as, natural causes like soil and rock erosion (Koçak et al., 2010; Beusen et al., 2016). In addition to diffuse pollution, the presence of nitrate and phosphate pollution indicates the existence of point pollution sources.

Similar to findings in this study, Beusen et al. (2016) reported that agricultural surface runoff is the primary source of N and P inputs to the ocean globally. Also, NE Med received a significant amount of mineral dust coming from Sahra, Middle East, and Arabian deserts (Guerzoni et al., 1999; Kubilay et al., 2000; Koçak et al., 2004). 90% of dissolved nitrogen and 40% of dissolved phosphate were obtained from atmospheric sources in NE Med (Koçak et al., 2010).

Nutrient Load Estimation

Similar to the nutrient concentrations, the monthly average nutrient load reaching the NE Med varied in time. Monthly average of NO_2, NO_3, NH_4 and PO_4 load varied between 0.07-31.2 ton/month, 15-1226 ton/month, 0.5-539 ton/month, 1-267 ton/month, respectively (Table 1).

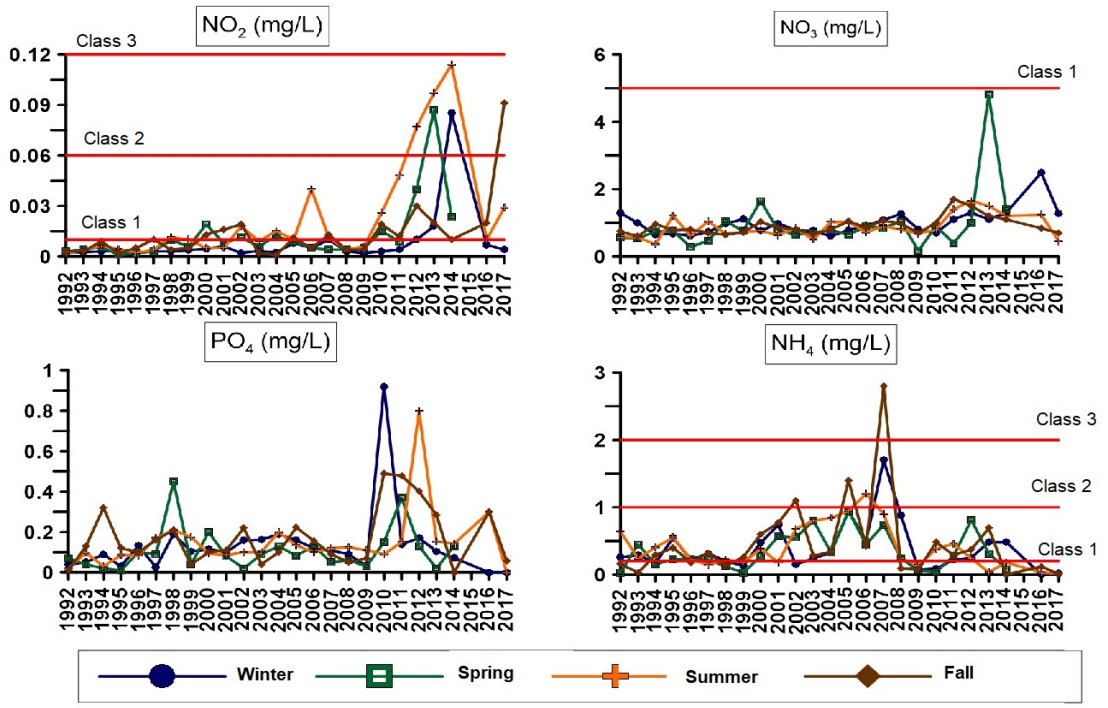

Figure 2. Comparison of nutrient concentration of Göksu River with surface water quality management regulation

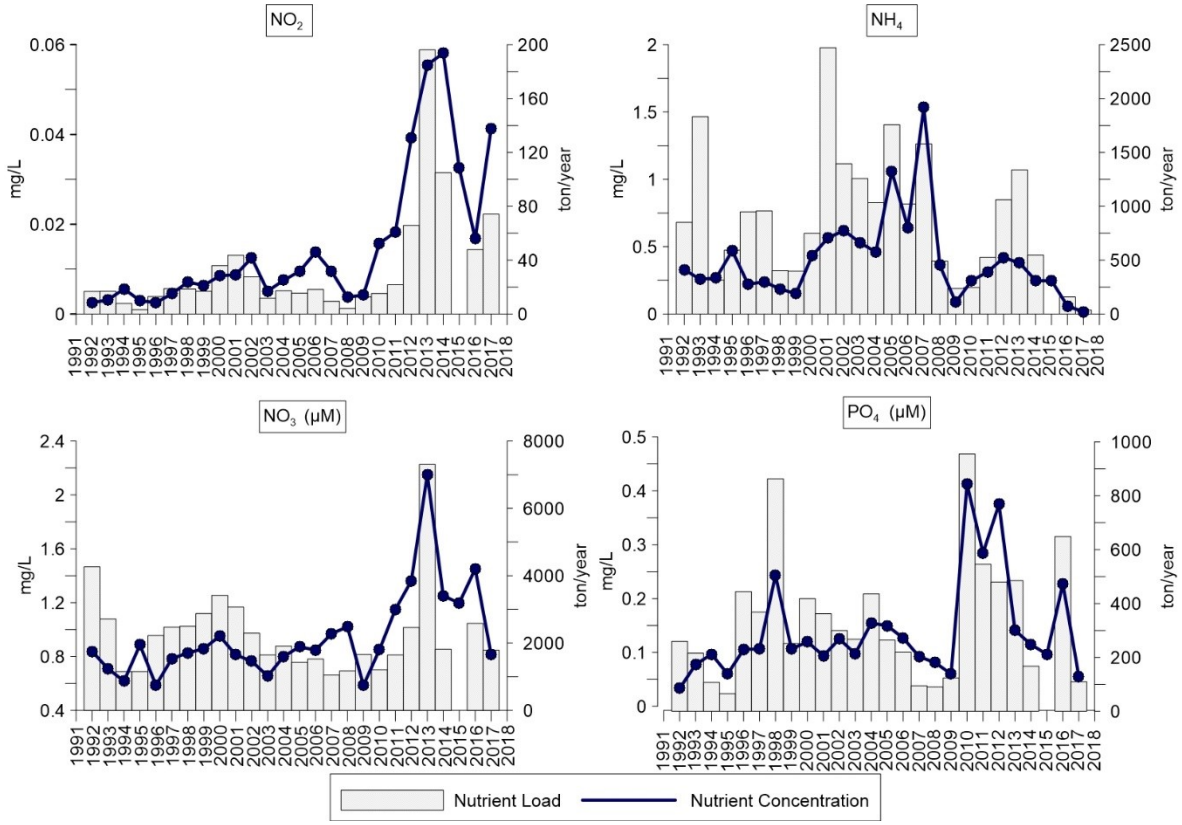

Figure 3. Annual nutrient load reaching to NE Med

Akçay & Tuğrul (2018) estimated the annual average NO_2, NO_3, NH_4, PO_4 load coming from Seyhan, Ceyhan, Berdan, Lamas, and Göksu Rivers as 966, 19 420, 2 796, 10 214 ton/year, respectively. In this study, the nutrient load coming from the Göksu River was estimated as 33 ton NO_2/year, 2.294 ton NO_3/year, 887 ton NH_4/year, and 342 ton PO_4/year (Table 1). As compared with the data from Akçay & Tuğrul (2018), the amount of NO_2, NO_3, NH_4, PO_4 coming from the Göksu River constitutes 3 %, 11 %, 32 %, and 33 % of the collected food load reaching the NE Med, respectively.

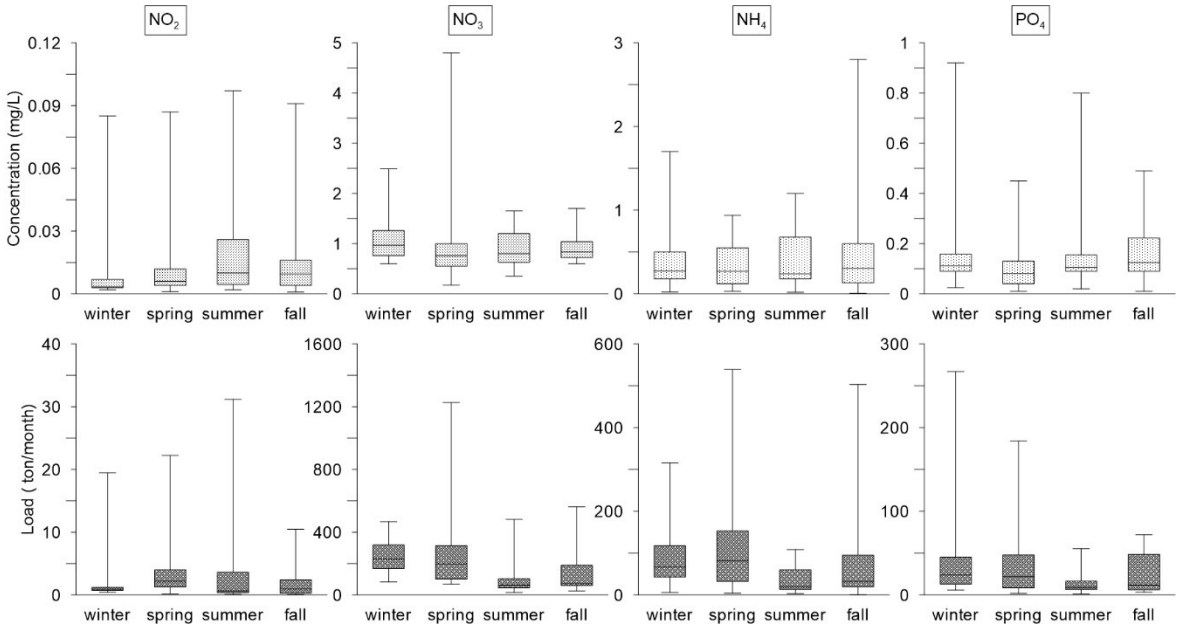

Figure. 4. Seasonal variation in nutrient load and nutrient concentration

Riverine nutrient load was estimated to be highest between 2012 and 2014. According to Eastern Mediterranean Climate Center database, precipitation observed in February and May of 2010-2013 was significantly greater than annual precipitation average of 1970-2010 (EMCC, 2020). This intense precipitation could increase the phosphate and nitragen load in the surface runoff considering the intense agricultural activity in the downstream of Göksu River (Figure 1).

Results revealed that nutrient load was increased with increasing nutrient concentration (Figure 3) throughout the sampling period as expected; since, both quality and quantity of surface runoff affect the nutrient load carried by rivers (Ackerman & Schiff, 2003). Also, both annual average load and concentration of NO_2, NO_3, and PO_4 were seen to be increasing between 2010 and 2015 which is coherent with the previous studies conducted in Cilician Basin (Akçay & Tuğrul, 2018; Kılıç et al., 2018).

Even though there was no statistically significant seasonal variation observed in both nutrient load and nutrient concentration ($p>0.05$), a high standard deviation was observed depending on seasons (Figure 4). In general, the highest nutrient concentrations observed in summer seasons due to significant evaporation, and the highest nutrient load observed in winter-spring when precipitation is dominant.

Satellite Images

The chemical composition of phytoplankton in the ocean is known as the Redfield ratio which is 106 C:16 N:1 P (Goldman, 1979).

Parameters which cause deflection from this ratio are referred to as limiting nutrient for growth (Redden et al., 2009). Göksu River contains a large amount of phosphate to sustain the growth; whereas, suffers from a lack of nitrogen (Figure 5). Therefore, high phosphate-containing waters of the Göksu River cause an increase in the primary productivity of NE Med where growth is mainly nitrogen-limited as a result of high N/P ratio (Krom et al., 1991; Koçak et al., 2010).

Figure 5. Redfield ratio of Göksu River

Satellite images representing the impact of the Göksu River on NE Med were also confirmed the relationship between river effluents and coastal waters (Figure 6). It is found that nutrient-rich waters of Göksu River transported along the coastline toward the west and southwest via Asian Minor current that results in the enrichment in the surface chl-*a* concentration of coastal zone. Also, chl-*a* concentration in the inshore area is increasing depending on nutrient load coming from the Göksu River.

Satellite images showed unique oceanology features of the NE Med which occupy a very important place in primary production in the region (Figure 6). During winter when nutrient-rich waters of deep waters were brought to the surface

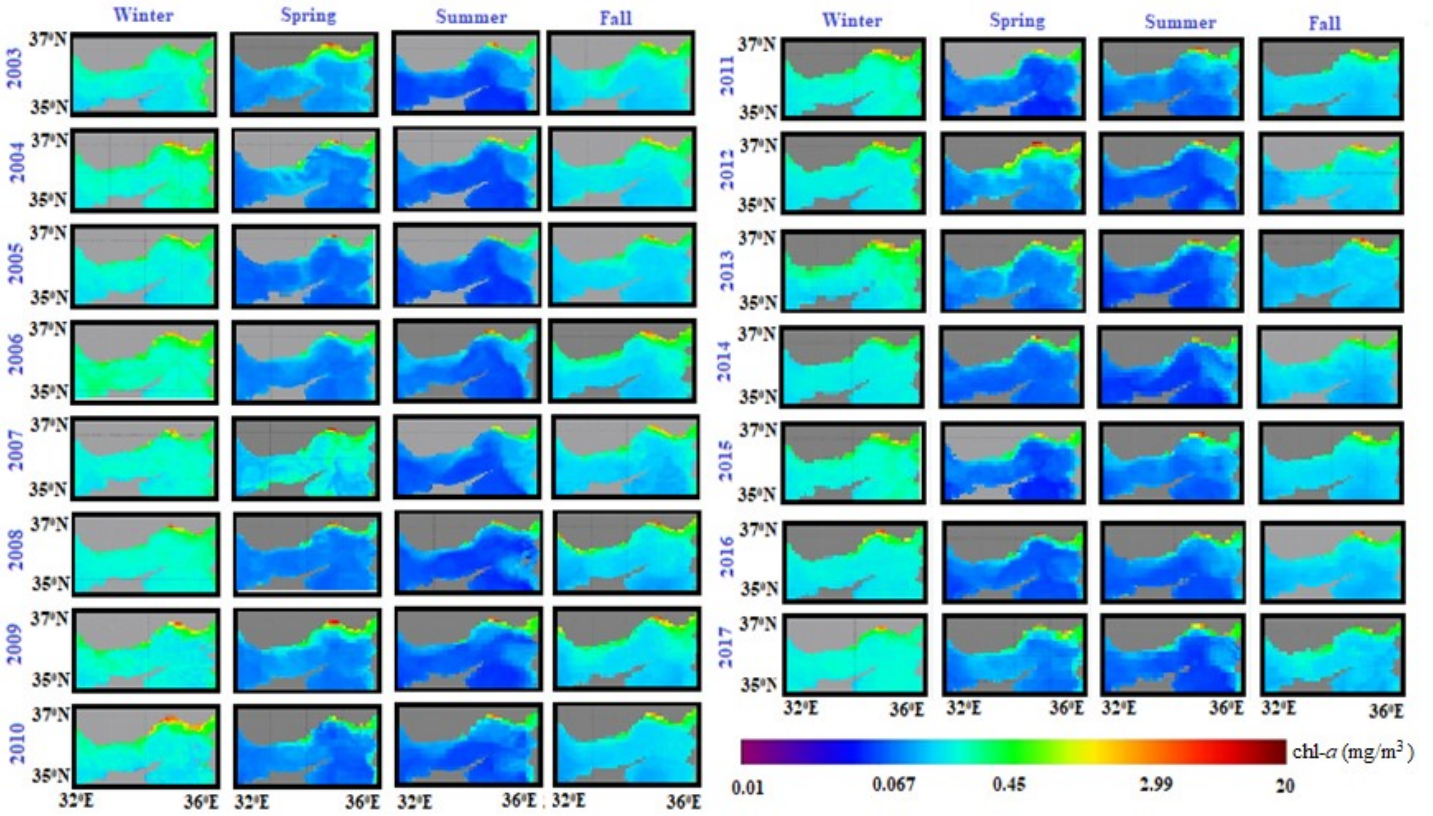

Figure 6. Seasonal surface chlorophyll-*a* concentration between 2003 and 2017

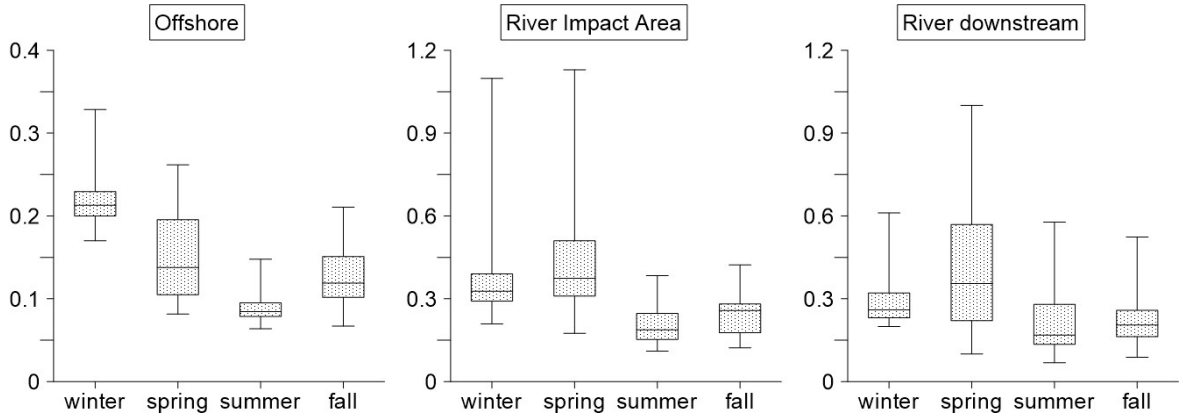

Figure 7. Seasonal variation in surface chlorophyll-*a* concentration

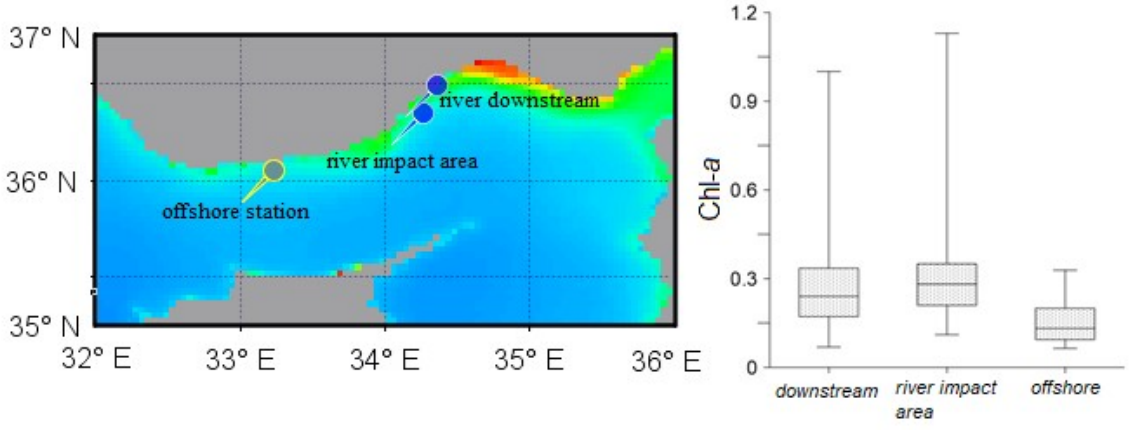

Figure 8. Variation of surface chl-*a* concentration (mg/m³) depending on station type

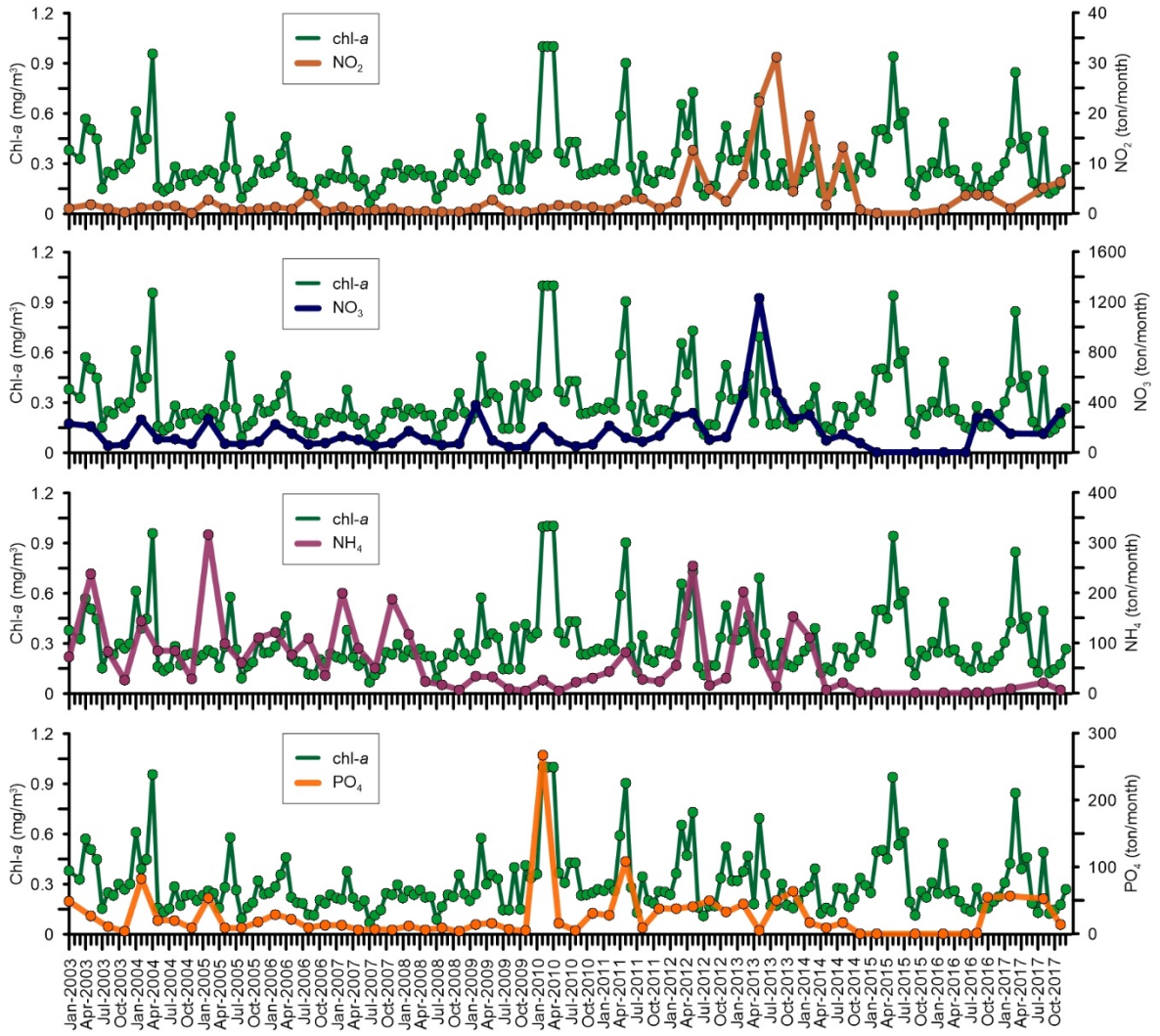

Figure 9. Relationship between nutrient salts and surface chl-*a* concentration

with mixing (Doğan Sağlamtimur & Tuğrul, 2008), surface chl-*a* concentrations were reached to the peak levels in the offshore station (Figure 6, Figure 7). On the contrary, strong summer stratification observed in the NE Med caused a strong decrease curve in the nutrient concentration in the euphotic zone (Latasa et al., 2017; Mena et al., 2019), which leads to the decrease in chl-*a* concentration in summer (Figure 6, Figure 7). Even though, these conditions were also valid for the river discharge area, increasing nutrient load as a result of increased precipitation alter the primary production dynamics. Nutrient rich-low salinity waters of Göksu River could not diffuse offshore due to blockage of Asian Minor current (Akpınar et al., 2016). As a result, chl-*a* concentration was increased in the river discharge area; whereas; it was still low in the offshore region (Figure 6). Therefore, as a consequence of physicochemical dynamics of NE Med, surface chl-*a* concentration was sorted from lowest to highest as winter>spring>fall>summer.

To understand the impact of Göksu River on the productivity of receiving environment, surface chl-*a* concentration in 3 different stations representing the river downstream (34.14 N, 36.43 E), river impact area (34.05 N, 36.25 E), and open waters (33.01 N, 35.85 E) were examined from satellite images (Figure 8).

The average surface chl-*a* concentration in the Göksu River downstream, impact area, and offshore area were determined as 0.29 mg/m^3, 0.31 mg/m^3, and 0.15 mg/m^3, respectively (Figure 8). In the river downstream phytoplankton have to adapt to the changing environmental conditions such as low salinity; while, in the river impact area phytoplankton grow under more stable physical conditions (Uysal et al., 2019). For that reason, the highest surface chl-*a* concentration was observed at the river impacted area. On the other hand, the lowest surface chl-*a* concentration was observed at the offshore station where chl-*a* concentration was decreased by more than 2 folds (Figure 8).

Besides, surface chl-*a* concentration at the Göksu River downstream and river impact area have a higher variation range than offshore waters (Figure 8). This indicates that surface chl-*a* concentration in the coastal zone was more sensitive to anthropogenic activities; whereas, variations in the offshore

Table 2. Results of Pearson correlation analysis

		Chl-*a* (mg/m³)	NO_2 (mg/L)	NO_2 (ton/month)	NO_3 (mg/L)	NO_3 (ton/month)	NH_4 (mg/L)	NH_4 (ton/month)	PO_4 (mg/L)	PO_4 (ton/month)
Chl-*a* (mg/m³)	r	1								
	s									
NO_2 (mg/L)	r	-0.06	1							
	s	0.659								
NO_2 (ton/month)	r	0.117	0.783(**)	1						
	s	0.387	0							
NO_3 (mg/L)	r	0.056	0.484(**)	0.510(**)	1					
	s	0.68	0	0						
NO_3 (ton/month)	r	0.273(*)	0.419(**)	0.675(**)	0.719(**)	1				
	s	0.04	0.001	0	0					
NH_4 (mg/L)	r	-0.199	-0.132	-0.115	-0.088	-0.125	1			
	s	0.137	0.329	0.395	0.514	0.355				
NH_4 (ton/month)	r	0.107	-0.111	0.09	-0.11	0.239	0.649(**)	1		
	s	0.426	0.413	0.506	0.417	0.073	0			
PO_4 (mg/L)	r	0.322(*)	0.042	-0.054	-0.029	-0.046	-0.105	-0.086	1	
	s	0.015	0.76	0.691	0.832	0.738	0.441	0.528		
PO_4 (ton/month)	r	0.592(**)	-0.071	0.044	-0.144	0.148	-0.159	0.106	0.760(**)	1
	s	0	0.602	0.747	0.29	0.277	0.241	0.436	0	

Note: (*) Correlation is significant at 0.05 level. (**) Correlation is significant at 0.01 level. Where *r*: Pearson correlation coefficient, *s*: significance.

zone mainly driven by climatic variations like winter mixing and summer stratification.

To evaluate the relationship between nutrient load coming from Göksu River and surface chl-*a* concentration in the downstream of Göksu River, Pearson correlation analysis was used. The results revealed a linear relationship between PO4 concentration from the river and surface chl-*a* concentration ($p<0.01$) and a statistically significant correlation between NO_3 concentration and surface chl-*a* concentration. On the other hand, there was no significant relationship detected between chl-*a* and NO_2 and NH_4 salts (Table 2, Figure 9).

The amount of NO_3 and NH_4 reaching the NE Med by wet or dry deposition is reported to be significantly higher than PO_4 (Koçak et al., 2010). Therefore, in P-limited NE Med, phytoplankton meets the PO_4 amount essential for growth from riverine inputs. In other words, a combination of P-rich waters of Göksu River with N-rich NE Med increases the primary productivity in the Göksu River impact area sharply. The linear relationship observed between chl-*a* and PO_4 load also confirm this relationship (Figure 9).

NE Med coasts are known with the large size phytoplankton (mainly diatom) blooms observed during rainy seasons when nutrient load coming from surface waters was significant (Siokou-Frangou et al., 2010; Yücel et al., 2017). It was reported that NO_3 uptake affinity of diatoms and pico-eukaryotes were comparatively higher than other phytoplankton groups (Painter et al., 2014; Moschonas et al., 2017). As a result, a significant relationship between NO_3 salt and surface chl-*a* concentration was observed during bloom seasons ($p<0.05$) (Table 2; Figure 9).

Interpolation-based average estimator model was reported as a sufficient tool in nutrient load calculations (Laznik et al., 1999; Stalnacke et al., 1999; Bettiol et al., 2005; Johnes, 2007; Buhvestova et al., 2011; Kılıç et al., 2018). Such models are particularly satisfactory in monitoring long-term seasonal trends and time-dependent changes (Johnes, 2007). However, underestimation and overestimation could be also possible due to many reasons. Firstly, it is possible to observe different nutrient concentrations depending on the river section (Johnes, 2007) or flow rate variation depending on season. Secondly, mass transport and transformation kinetics were related to the many environmental constraints which are usually hard to interpret (Kılıç et al., 2018). Lastly, some uncertainties were also possible in using seasonal data to estimate annual nutrient load since it also may cause deflection from the actual load. In order to overcome these existing uncertainties, it is necessary to ensure the accuracy, effectiveness of the monitoring program, and the accuracy of the statistical method in which the nutrient load is calculated (Stalnacke et al., 1999). Long-term (1992-2017) monitoring results from the national monitoring program were used to ensure the accuracy of the data obtained in this study.

Conclusion

This study is conducted to evaluate the impact of the nutrient load of the Göksu River on the NE Med. Monthly average NO_2, NO_3, NH_4 and PO_4 load varied between 0.07-31.2 ton/month, 15-1226 ton/month, 0.5-539 ton/month, 1-267 ton/month, respectively. Even though, there was no statistically significant seasonal variation observed in both nutrient load and nutrient concentration ($p>0.05$), a high standard deviation was observed depending on season. The greatest nutrient load observed in the winter-spring season lead to an increase in primary productivity the downstream of Göksu River. Surface chl-*a* concentration showed more than a two-fold decrease from inshore to an offshore area that proves the positive impact of the Göksu River. The linear relationship between discharged PO_4 load and surface chl-*a* concentration was detected which represents the positive impact of the combination of P-rich waters of Göksu River with N-rich NE Med ($p<0.01$). Also, a significant relationship observed between discharged NO_3 load and surface chl-*a* concentration ($p<0.05$) was represent the impact the phytoplankton blooms observed during the winter-spring season.

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Compliance With Ethical Standards

Authors' Contributions:

EK and NY designed the study. EK conducted the necessary calculations, data processing and statistical applications. NY advises the processing of study and contributes to discussion of obtained data. Both authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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RESEARCH ARTICLE

Measuring mental workload and heart rate variability of officers during different navigation conditions

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ABSTRACT

Mental workload (MWL) has a negative effect on the functional states of watchkeeping officers that ultimately causes collisions and groundings at sea. The aim of this study is to measure the MWL of officers during different navigation conditions. This study was conducted in a bridge simulator with 11 participants. Heart rate variability (HRV) measurements were taken during the 4 steps which have different difficulty levels and subjective assessments were taken at the end of each step by using NASA-TLX. The results of the measurements showed that different levels of navigation tasks caused significantly different levels of MWL and HRV values and MWL and HRV increased when task difficulty increased. Additionally, the correlation between MWL perceived by the participants and the heart rate variability values of the participants was found statistically significant. This study provides an example of predicting MWL for routine navigation operations by using physiological measures in maritime transportation.

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Introduction

While human error is the primary contributor of accidents where about 85% of all accidents were caused by human error (Kurt et al., 2016), it was stated that 16% of collisions, 30% of groundings were related to mental workload (MWL) of watchkeeping officers (Akhtar & Utne, 2015) in furtherance the determination that technology and automation have reduced the number of crew and increased the workload of officers (Louie & Doolen, 2007; Grech et al., 2008). This indicates that human element related issues will continue to be one of the major issues in marine transportation assets.

The workload is defined simplistically as a demand placed upon humans. Demand is specified by the aim of task performance. Therefore, the workload is the effect of demand on the individual in terms of stages used in energetics and information processing. More specifically, the workload is the amount of information processing capacity used for task

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performance (De Waard, 1996). Kahneman (1973) argued that in the cognitive system, difficult and complex tasks increase arousal levels, providing additional resources to cope with these tasks. In the light of this information, MWL can be monitored with the aid of physiological data collection in terms of autonomic nervous system activation. Kahneman (1973)'s approach, in terms of being measurable, was not considered sufficient alone but has been adopted by other researchers (De Waard, 1996; Young & Stanton, 2002).

MWL causes changes in human performance and behaviour that are nearly related to the physiological and biochemical changes in the body which are based on humoral regulation, nervous regulation and autoregulation (Lean & Shan, 2012). The cardiovascular reaction is one of these physiological changes. Heart rate variability (HRV) is a useful feature of cardiovascular activity and has successful classification accuracies in MWL and stress levels (Alberdi et al., 2016). HR increases when task demand increases (De Waard, 1996; Backs et al., 2000; Embrey et al., 2006; De Rivecourt et al., 2008), in multi task conditions (Fournier et al., 1999), during additional memory load (Finsen et al., 2001), when requiring problem solving (Splawn & Miller, 2013) or stressful condition increases (Sharma & Gedeon, 2012; Alberdi et al., 2016), HR increases and HRV decreases (De Waard, 1996; Embrey et al., 2006; Sharma & Gedeon, 2012; Alberdi et al., 2016). On the other hand, the increase of HRV was stated in high complexity tasks for longer durations (Fairclough et al., 2005; Gao et al., 2013). HRV metrics include the time-domain, frequency domain, time-frequency and non-linear analysis (Selvaraj et al., 2008; Ramshur, 2010; Aimie-Salleh et al., 2019). Frequency domain analysis has been mostly used in MWL studies. A decrease in mid (0.07-0.14 Hz) and high frequencies (0.15-0.50 Hz) is associated with an increase in mental effort and task demand (Veltman and Gaillard, 1998). The increase of LF/HF by the increase of LF (0.02 -0.06 Hz) together with the decrease of HF is associated with MWL (Lean & Shan, 2012) and stress (Sharma & Gedeon, 2012; Alberdi et al., 2016). However, the decrease of LF in high task difficulty was stated by authors (Delaney & Brodie, 2000; Lehrer et al., 2010; Splawn & Miller, 2013).

Mental workload measurement is not an issue widely studied in the maritime domain, compared to other industries such as aviation, railway, car driving etc. (Özsever & Tavacıoğlu, 2018). In maritime human factor research, there are several data collection methods related to mental workload or fatigue. These are physiological, physical (eye movement etc.), environmental measures, performance analysis in simulator environment, interviews, questionnaires, observations and logbooks, accident/incident analysis and computer-aided design/evaluations (Grech et al., 2008). Commonly, physiological-physical, subjective and performance measures have been used in workload measure studies (Embrey et al., 2006). The studies conducted in recent years have focused on the MWL measurements in some maritime-specific tasks. Wu et al. (2017) associated the EEG and the HRV data, obtained from 10 participants in engine control room simulator, with MWL as task difficulty increased. Orlandi & Brooks (2018) applied a similar method to ship pilots and reached similar results. Yan et al. (2019) used eye response measurement to predict MWL for engine department tasks.

This study aims to measure the mental workload of officers during different navigation conditions, adopting the self-reported and HRV measures. The following hypothesis of this study is mainly studied:

- Different levels of navigation tasks should draw out different levels of MWL and HRV values.

Methods

A total of 11 participants (5 female) were recruited to perform navigation scenarios in a bridge simulator in this study. At least, participants must have had an Oceangoing Watchkeeping Officer certificate and one contract sea experience as an officer in merchant ships. The mean age was 28.4 (SD=4.8) and the mean period of service of participants was 12.4 months (SD=7.9). The study was conducted in a bridge simulator (Figure 1) of Piri Reis University (İstanbul, Turkey) with navigation tasks based on the Malacca Straight passage (Figure 2).

Figure 1. Bridge simulator

Navigation scenarios have been varied being used at different levels of difficulties in mostly visibility, traffic density and geography parameters. Gould et al. (2009) used the variables like geography, visibility and traffic density for

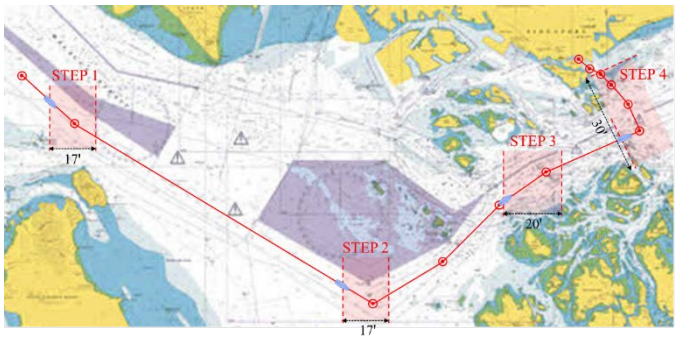

Figure 2. Navigation area used in the simulator with route legs and measurement areas as stated in steps. Image obtained from Admiralty Chart BA 3833

navigation scenarios with 4 different levels of difficulty Collision threat, target behaviour and traffic were used as variables for navigation scenario, which was conducted as 6 minutes and 18 times, in another study (Robert et al., 2003). Similar to the study of Gould et al. (2009), visibility, traffic density, geography, equipment condition and speed restriction were determined as difficulty variables in the study of Grabowski & Sanborn (2003). In this study, the difficulty level of the navigation scenario was gradually adjusted according to traffic density, visibility and geography by combining in 4 steps as:

- Step 1; high visibility, low traffic density, easy geography
- Step 2; high visibility, moderate traffic density, easy geography
- Step 3; moderate visibility, high traffic density, moderate geography
- Step 4; low visibility, high traffic density, hard geography

Heart rate measurement was taken from the participants during the navigation where measurement areas are stated in Figure 2. An optical pulse sensor was used in this study, which measures the photoplethysmogram (PPG) signal from a finger to estimate heart rate. This measurement is used to evaluate the PPG signal and to convert the PPG signal to heart rate. This unit contains electronics attached to a velcro cuff for a finger with a cable length of 9 inches (Figure 3a). The sampling rate was 128 Hz. The ConsensysPRO software was used to convert PPG data to heart rate and IBI (Interbeat interval) signal (Figure 3b) ("Optical Pulse Sensor User Guide," 2016).

The following HRV features to be used in statistical analysis have been extracted from the IBI signal:

- Standard deviation of NN intervals (SDNN) (Eq. (1)),
- Triangular interpolation of IBI interval histogram (TINN) (Eq. (2)),
- Poincaré plot standard deviation along the line of identity (SD2),
- Absolute spectral power of low frequency (0.04-0.15 Hz) (aLF), absolute spectral power of high frequency (0.15-0.4 Hz) (aHF) and the ratio of low frequency to high frequency (LF/HF) in time-frequency Lomb-Scargle Periodogram (Eq. (3)) domain.

$$\mathrm{SDNN} = \sqrt{\frac{1}{N-1}\sum_{n=1}^{N}[NN_n - mean(NN)]^2} \quad (1)$$

$$\mathrm{TINN} = M - N \quad (2)$$

where N is total window length and NN is the normal-to-normal time interval (Aimie-Salleh et al., 2019). M and N values represent the minimum and maximum values of a triangle which is shaped on the IBI histogram graphic, on the time axis (Ramshur, 2010).

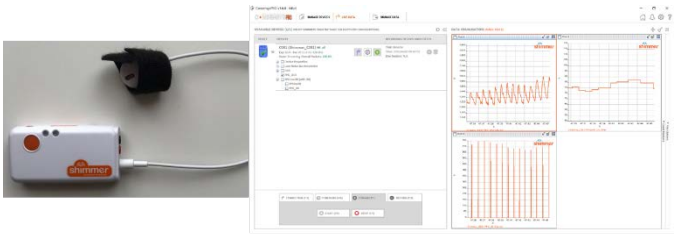

Figure 3. Optical Pulse Sensor (a) and recording the PPG data (b)

Min-max normalization was applied in order to eliminate individual differences between the subjects and to observe the physiological change during the tasks. The equation (4) was performed for normalization;

To compare the HRV results of the participants with their MWL levels, NASA Task Load Index (NASA-TLX) was used at the end of each step of the navigation scenario by the participants.

Results

All statistical analysis has been performed in SPSS v24. The NASA-TLX scores of each step evaluated by the participants have been statistically analysed and summarized in Table 1. Figure 4 shows the boxplots of the distribution of total scores among 4 steps.

ANOVA has been also performed to show differences in the results of HRV features in different difficulty levels of navigation scenarios. Table 2 presents the ANOVA results of HRV features. Figure 5 shows the means plots of HRV features among 4 steps.

To analyse the relation between NASA-TLX scores and HRV features statistically, correlation analysis has been performed (Table 3). It is possible to observe how HRV features except LF/HF were positive significantly correlated with NASA-TLX scores of the participants.

Table 1. Analysis of variance (ANOVA) of NASA-TLX scores among 4 navigation steps

	Step 1 (*M* ± *SD*)	Step 2 (*M* ± *SD*)	Step 3 (*M* ± *SD*)	Step 4 (*M* ± *SD*)	*p*
Mental demands	3.57 ± 2.23	9.94 ± 4.11	13.88 ± 5.81	20.64 ± 6.27	<0.001**
Performance	5.90 ± 5.31	4.79 ± 2.76	6.30 ± 3.87	10.27 ± 5.19	0.031*
Temporal demands	1.00 ± 1.25	6.64 ± 7.39	9.70 ± 7.56	15.54 ± 10.40	0.001**
Efforts	3.47 ± 2.52	6.03 ± 3.83	9.39 ± 5.13	14.00 ± 5.35	<0.001**
Frustration	1.67 ± 1.29	6.27 ± 6.18	6.88 ± 5.43	12.12 ± 9.17	0.006**
NASA-TLX score	15.60 ± 8.93	33.67 ± 16.22	46.15 ± 14.28	72.58 ± 10.57	<0.001**

Note: * indicates significance level is 0.05; ** indicates significance level is 0.01.

Table 2. ANOVA of HRV features among 4 navigation steps

	Step 1 (*M* ± *SD*)	Step 2 (*M* ± *SD*)	Step 3 (*M* ± *SD*)	Step 4 (*M* ± *SD*)	*p*
SDNN	0.20 ± 0.24	0.45 ± 0.16	0.50 ± 0.16	0.56 ± 0.17	<0.001**
TINN	0.26 ± 0.22	0.46 ± 0.21	0.48 ± 0.16	0.54 ± 0.14	0.008**
SD2	0.20 ± 0.24	0.45 ± 0.15	0.49 ± 0.16	0.56 ± 0.17	<0.001**
aLF	0.18 ± 0.22	0.37 ± 0.11	0.40 ± 0.19	0.50 ± 0.12	0.001**
aHF	0.19 ± 0.23	0.39 ± 0.14	0.46 ± 0.17	0.47 ± 0.19	0.006**
LF/HF	0.33 ± 0.23	0.45 ± 0.14	0.41 ± 0.15	0.54 ± 0.11	<0.033*

Note: * indicates significance level is 0.05; ** indicates significance level is 0.01.

Table 3. Correlations between NASA-TLX scores and HRV features

		NASA-TLX	SDNN	TINN	SD2	aLF	aHF	LF/HF
NASA-TLX	Pearson Corr.	1	0.401**	0.321*	0.416**	0.390**	0.332*	0.221
	Sig. (2-tailed)		0.008	0.036	0.006	0.01	0.029	0.154
	N	43	43	43	43	43	43	43
SDNN	Pearson Corr.	0.401**	1	0.726**	0.997**	0.834**	0.841**	0.467**
	Sig. (2-tailed)	.008		0.000	0.000	0.000	0.000	0.002
	N	43	43	43	43	43	43	43
TINN	Pearson Corr.	0.321*	0.726**	1	0.740**	0.683**	0.595**	0.507**
	Sig. (2-tailed)	0.036	0.000		0.000	0.000	0.000	0.001
	N	43	43	43	43	43	43	43
SD2	Pearson Corr.	0.416**	0.997**	0.740**	1	0.837**	0.825**	0.490**
	Sig. (2-tailed)	0.006	0.000	0.000		0.000	0.000	0.001
	N	43	43	43	43	43	43	43
aLF	Pearson Corr.	0.390**	0.834**	0.683**	0.837**	1	0.789**	0.672**
	Sig. (2-tailed)	0.01	0.000	0.000	0.000		0.000	0.000
	N	43	43	43	43	43	43	43
aHF	Pearson Corr.	0.332*	0.841**	0.595**	0.825**	0.789**	1	0.391**
	Sig. (2-tailed)	0.029	.000	0.000	0.000	0.000		0.000
	N	43	43	43	43	43	43	43
LF/HF	Pearson Corr.	0.221	0.467**	0.507**	0.490**	0.672**	0.391**	1
	Sig. (2-tailed)	0.154	0.002	0.001	0.001	0.000	0.000	
	N	43	43	43	43	43	43	43

Note: * indicates significance level is 0.05; ** indicates significance level is 0.01.

$$P_{LS}(f) \equiv \frac{1}{2\sigma^2}\left\{\frac{\left[\sum_{n=1}^{N}(X(t_n)-\bar{x})cos(2\pi f(t_n-\tau))\right]^{2^\circ}}{\sum_{n=1}^{N}cos^2(2\pi f(t_n-\tau))}+\frac{\left[\sum_{n=1}^{N}(X(t_n)-\bar{x}_2)sin(2\pi f(t_n-\tau))\right]^{2^\circ}}{\sum_{n=1}^{N}sin^2(2\pi f(t_n-\tau))}\right\} \quad (3)$$

where $\tau \equiv tan^{-1}\left(\frac{\sum_{n=1}^{N}sin(4\pi f t_n)^\circ}{\left(\sum_{n=1}^{N}cos(4\pi f t_n)^\circ\right)^\circ}\right)$, $\bar{x}$ and σ^2 are the mean and variance of the time series (Ramshur, 2010).

$$\psi'_{j\_normalized} = \frac{\psi_{j,t}{}'^{\circ} - {}^{\circ}\psi_{j,min}{}'}{\psi_{j,max}{}'^{\circ} - \psi_{j,min}{}'} \quad (4)$$

where $\psi'_{j,min}$ and $\psi'_{j,max}$ are the minimum and maximum values of related extracted features within the measured data of the subject.

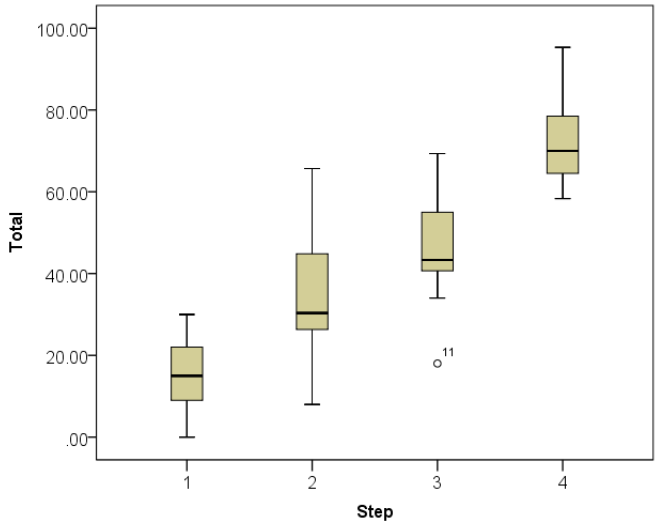

Figure 4. Boxplot of NASA-TLX total scores among 4 navigation steps

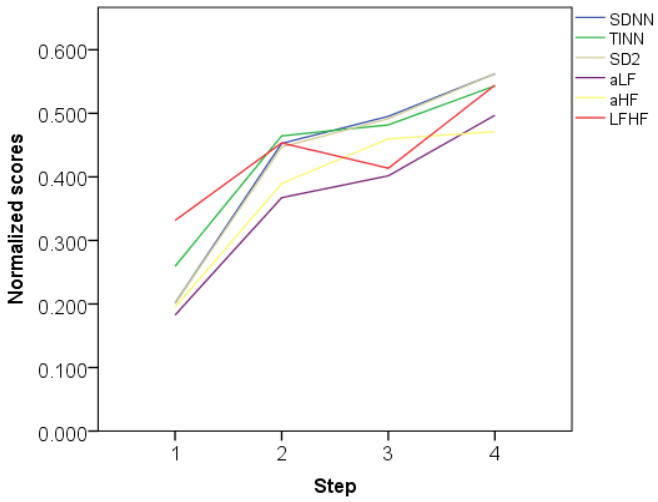

Figure 5. Means plots of HRV features among 4 navigation steps

Discussion

Different levels of navigation tasks caused different levels of MWL and HRV values. ANOVA results show that there are significant differences in the NASA-TLX scores of 5 different dimensions and in total, among 4 steps which have different difficulty levels, i.e., MD (p<0.01), P (p<0.05), TD (p<0.01), E (p<0.01), F (p<0.01) and total (p<0.01). It was observed that MWL perceived by the participants increased when task difficulty increased. It can be seen also that there are significant differences in the HRV features among 4 steps which have different difficulty levels, i.e., SDNN (p<0.01), TINN (p<0.01), SD2 (p<0.01), aLF (p<0.01), aHF (p < 0.01) and LF/HF (p<0.05). The increase of HRV features except aHF were found significant similar to the results of previous studies in that the increase of HRV was stated in high complexity tasks for longer durations (Fairclough et al., 2005; Gao et al., 2013). aHF was expected to decrease when task difficulty increased according to the studies in the literature.

The correlations between NASA-TLX and HRV features except LF/HF were also found significant. The reason that NASA-TLX scores were not correlated with LF/HF can be explained as increasing of aHF together with aLF. This result couldn't support the literature that increase of LF/HF by the increases of LF together with the decrease of HF is associated with MWL (Lean & Shan, 2012). However, LF/HF increased when task difficulty increased but this increase couldn't be found statistically significant when comparing the increase of NASA-TLX scores.

The increase of HRV time-domain features (SDNN, TINN) (this means the decrease of heart rate) in this study has been not observed in other maritime-related studies. Heart rate increased when task difficulty increased in ship manoeuvring (Orlandi & Brooks, 2018), heart rate increased and HRV time-domain features decreased when task difficulty increased in engine department tasks (Wu et al., 2017). On the other hand, the increase of LF/HF in this study was found similar to the results of the related studies in the literature. However, the change of all HRV features has not been found statistically significant in both maritime-related studies.

In general, the changes in NASA-TLX scores and time and frequency-based HRV features were found to be significant due to increased task load. The results of this study were found to be similar and significant to the results of the studies in the literature. Differently from other maritime-related studies in literature, the effect of increasing task load in routine navigation tasks on officers of the watch was shown with empirical data.

Conclusion

It is known that the human factor has a major effect on maritime casualties that cause great harm to the environment, economy and maritime sector. It was stated that while human error is the primary contributor to accidents, a good part of collisions and groundings were related to the mental workload (MWL) of watchkeeping officers. This study focused on the MWL measurement in routine navigation tasks instead of the studies conducted in recent years, which have focused the MWL measurements in the engine department and ship manoeuvring tasks. Therefore, the main aim of this study was to measure the mental workload of officers during different navigation conditions. The results of the measurements showed that different levels of navigation tasks caused significantly different levels of MWL and HRV values and MWL and HRV increased when task difficulty increased. Consequently, this study will contribute to the literature in terms of predicting

MWL for routine navigation operations by using physiological measures in maritime transportation.

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Compliance With Ethical Standards

Authors' Contributions:

BÖ: Taking the measurements, Data handling, Writing-original draft.

LT: Resources, Writing-review & editing.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

This study was approved by the Medical and Engineering Sciences Human Research Ethics Committee of Istanbul Technical University.

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RESEARCH ARTICLE

The use of S-GnRHa (salmon gonadotropin releasing hormone analogue) in induced breeding and early embryonic development of Gulsha, *Mystus cavasius*

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M. cavasius

ABSTRACT

The current study was carried out to optimize the dose of the synthetic hormone for induction, and to observe the embryonic and larval developmental in Gulsha, *Mystus cavasius*. Induced breeding was conducted by using Ovupin (S-GnRHa) hormone (each 1.5 ml vial contain 0.2 mg of an analogue of S-GnRHa) at four different doses i.e., 0.25, 0.5, 1.0- and 1.50-ml kg^{-1} body weight (BW) for females, and the half of these doses were applied to males. Among the applied doses, 0.5 ml kg^{-1} BW for female and 0.25 ml kg^{-1} BW for male provided the maximum fertilization (83.66%) and hatching (80.0%) rates. The eggs of *M. cavasius* were strongly adhesive, with covering on egg surface. The average diameter of fertilized eggs just after spawning was 85.58±5.87 µm. After fertilization, the first, second, and third cleavage stages occurred within 20-25, 35-40 and 60-65 min, respectively. The identity of blastomeres was gradually lost and appeared at 64-cell stage to 128-cell stage onwards. The 64-cell stage appeared at 150-160 min and the morula stage 3:00-3:20 h (blastomeres completely lost), respectively. The gastrula stage appeared at 5:0-5:30 h in which the blastoderm spread in both the sides covering about 60-70% area, together with a thread-like germinal ring. Afterward, twisting locomotion was recorded at 23:30 h. The larvae started hatching at 24:00 to 25:00 h. The barbells were partially visible when the larvae were 10-12 h of age. Finally, the yolk sac was fully absorbed in the end of Day 3.

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Introduction

Mystus cavasius (Order- Siluriformes), locally known as Gulsha, is a widespread catfish in Bangladesh (Chakrabarty & Ng, 2005; Rao, 2017) and it has been drawing interest due to its premium taste and nutritional prominence (Latif et al., 2018). The fish can easily be recognized by its elongated and compressed body, four pairs of barbells and a serrated spiny dorsal fin. Most of the catfish are highly preferred by local customers for their less intermuscular bone and flavor (Gupta, 2015; Jabed et al., 2020). Now, it is a vulnerable Small Indigenous Species (SIS) in Bangladesh (Iqbal et al., 2015; IUCN Bangladesh, 2015).

Aquaculture in Bangladesh is an increasing sector and its species ranges are expanding day by day (Alam et al., 2014; Jayasankar, 2018; Alam et al., 2020). Fry and fingerlings from the wild sources, i.e., rivers, lakes, etc. are not adequate and reliable (Verma et al., 2017; Mishra et al., 2018; Müller et al., 2020). That is why the number of hatcheries is also increasing to fulfil the demand of fry in aquaculture**.** The artificial propagation of Gulsha involves the injection of sexually mature females and males with synthetic hormones, with modifications, that have been used to spawn an entire range of catfish. Generally, catfish are injected with hormones under the peritoneal cavity (Bhenila & Biswas, 2014; Kumar et al., 2018; Kumar et al., 2021). Different hormones are used in different doses for different catfish species, which might have resulted in lower hatching and survival rates, and poor-quality fry and fingerlings. Generally pituitary gland (PG) extract was not used in the hatchery, especially for catfish as it is required in high amounts and the number of eggs ovulated is low (Mondol et al., 2014; Aktar, 2015). Moreover, the rearing techniques of embryonic, larval and fry stages of Gulsha are not well studied yet to increase the survival rates and produce quality fry and fingerlings. For artificial propagation studies; (i) it is important to identify the appropriate hormones and their dosage, (ii) to know the embryonic and larval stages of early fish development and (iii) to optimize the rearing techniques for fry to have quality seeds of Gulsha. For seed production, the early life history information should be known well to rear fry in the nursery ponds as it is related to maturity of gravid fish and yolk sac completion (Shehu et al., 2018; Zadmajid et al., 2019). In this study, the induced breeding techniques in hatchery condition for Gulsha were investigated to optimize dose of S-GnRHa and to observe the embryonic and larval developmental stages under microscopy.

Materials and Methods

Location of the Study

The experiments were conducted for a three-month period from June to August 2019 in two different locations: (a) the private hatchery at Alalpur, Mymensingh and (ii) the laboratory facilities of the Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh, Bangladesh. The induced breeding and fry rearing of *M. cavasius* were performed in Alalpur hatchery complex and the micrographs of embryonic development stages were taken at the Fisheries Biology and Genetics (FBG) Laboratory.

Designing the Experiments and S-Gnrha Injection

The cemented cisterns were washed properly prior to shifting the brood male and female for strictly maintaining hygienic and germ-free condition for spawning. The cemented cistern was washed by using lime and brushed. The male and female brood of *M. Cavasius* were collected from the hatchery pond for breeding. The brood fish were separated as male, and female based on their secondary sexual characteristics. Then, the selected brood fish were instantly transferred to the hatchery and located in the cistern for about 8h (from 8:30 am to 4:30 pm). They were kept under water showering that has both outlet and inlet facilities to induce the breeding conditioning. The dirt due to fish body mucous and the excreta of fish were washed out through the outlet because of continuous waterflow. The fish were left fasted while conditioning. A synthetic form of induced hormone, ovupin (S-GnRH) manufactured by Sansheng Pharmaceutical Co., Ltd, China, was purchased from the local market. Following the manufacture's instruction, sterilized distilled water was mixed with the hormone powder to make the solution for injection and each 1.5 ml vial contain 0.2 mg of an analogue of S-GnRHa (see Table 1 for the doses applied). After hormone preparation it was carefully taken into a 0.5 ml hypodermic syringe and injected intramuscularly in near the muscle area of lateral line. Injected fish were kept in the breeding cistern tanks with continuous water flow and monitored until they ovulate. Males and females were included in four different treatment groups, and in each group males and females were injected with specific doses of S-GnRHa (Table 1). For each treatment, there were three replications. The ratio of male to female was 1:1 in all treatments. There was no control group had been assigned as S-GnRHa hormone has been already used in induced breeding of *M. cavasius* and this research work was aimed at optimizing dose for commercial aquaculture.

Table 1. Induce breeding trial of *M. cavasius* with S-GnRHa hormone

Treatment	Hapa	Body Weight (g)		Dose (ml/kg) body weight	
		Female	Male	Female	Male
T1	H1, H2 H3	28.33±1.53	18.00±2.00	0.25	0.125
T2	H1, H2 H3	30.00±2.65	17.67±1.53	0.50	0 .25
T3	H1, H2 H3	30.00±0.82	18.33±1.70	1.00	0.50
T4	H1, H2 H3	26.00±3.61	16.67±1.53	1.50	0.75

Monitoring Embryonic and Larval Development and Water Quality Measurements

The hormone injected female and male fishes were kept into breeding cistern having a size of 1.5 × 0.5 × 1 m^3 with continuous water flow and once ovulation occurred, ovum was collected and transported to nearby facilities of the Bangladesh Agricultural University (BAU) for further analysis and observation of embryonic stages. Water temperature, pH and dissolved oxygen were analyzed on spot following standard methods (APHA, 1998). The developmental stage of the eggs was photographed under microscope (OLYMPUS CX21) with camera attachment (Rigla-32, Optikam B3 Digital camera, Italy). The water quality parameters i.e., water temperature, pH, and water transparency of the nursery pond were frequently recorded using Secchi's disk and measuring tape, pH paper, and thermometer.

Determination of Ovulation Rate, Fertilization Rate and Hatching Rate

The ovulation rate, fertilization rate and hatching rate was determined using the following equations (Eq. 1-3) from Legendre (2000), Rahman & Samat (2020) and Unuma et al. (2004), respectively.

$$Ovulation\ rate\ (\%) = \frac{Number\ of\ fish\ ovulated}{Total\ number\ of\ fish\ injected} \times 100 \quad (1)$$

$$Fertilization\ rate\ (\%) = \frac{Number\ of\ fertilized\ eggs}{Total\ number\ of\ eggs} \times 100 \quad (2)$$

$$Hatching\ rate\ (\%) = \frac{Number\ of\ eggs\ hatched}{Total\ number\ of\ fertilized\ eggs} \times 100 \quad (3)$$

Statistical Analysis

A one-way analysis of variance (ANOVA) implemented in MS Excel was used to determine whether there are any statistically significant differences between the different hormonal treatment groups. The level of significance of the results were tested following Duncan's and Turkey using IBM SPSS v27 program.

Results and Discussion

Artificial fertilization, together with embryonic development, was performed successfully, where 0.5 ml kg^{-1} BW for female and 0.25 ml kg^{-1} BW for male among four different doses showed the maximum fertilization and hatching rates, and significantly different ($P < 0.05$) from the doses of 0.25 ml kg^{-1} BW for female and 0.125 ml kg^{-1} BW for male (Table 1). Current study findings resembled to Mondol et al. (2014), where 0.5 ml kg^{-1} BW of ovupin for female was successful in carp seed production, whereas comparatively lower dose of 0.25 ml kg^{-1} of Ovaprim for females increased the effectiveness of spawning induction of blackfin sea bream, *Acanthopagrus berda* (Abbas et al., 2019). However, Araf et al. (2021) obtained relatively higher dosages of S-GnRHa i.e., 1-2.5 ml/kg body weight have been efficient in the spawning induction of air stinging catfish. All females were ovulated when treated with all the four different hormone doses. Mondol et al. (2014) also observed very similar results using ovupin in major carp species and Alam et al. (2006) using Ovaprim in estuarine catfish, *Mystus gulio*. Ali et al. (2014) obtained 82.67% ovulation rate with HCG and PG injection in *Heteropneustes fossilis*, El-Hawarry et al. (2016) got 70.76% with GnRHa plus domperidone (Dom) treatment in African catfish (*Clarias gariepinus*) and Hossen et al. (2021) acquired hatching success between 74.33-83.89% in *Mystus gulio* by using synthetic GnRHa. An account of 62% fertilization rate and 60% hatching rates have been reported for *M. cavasius* injected with 0.2 ml/kg BW ovatide (Das et al., 2018).

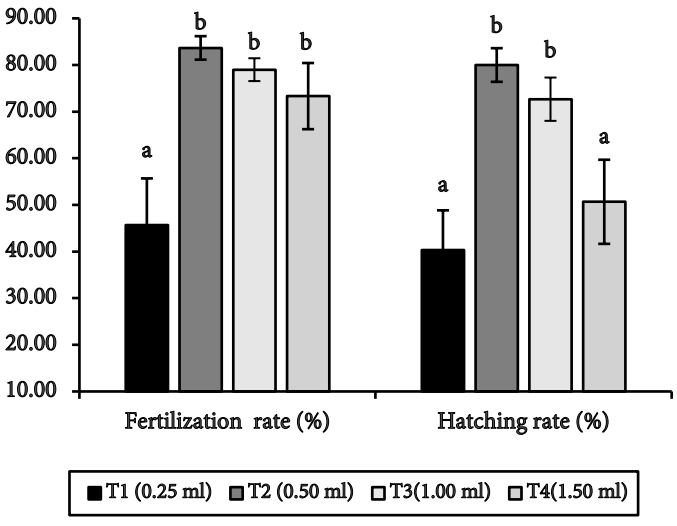

Figure 1. Effect of different doses of hormone on fertilization and hatching rate of *M. cavasius* (Y axes represent the percentages value of different parameters of Y axis; different letter in bar diagram refers the significant differences among the treatment)

Fertilization rate of eggs in different dosages i.e., 0.25, 0.5, 1.0 and 1.50 ml kg^{-1} BW were 45.66±10.01%, 83.66±2.51%, 79.00±3.00% and 73.33±7.09%, respectively (Figure 1). Mosha (2018) obtained highest fertilization rate of 87.34% in African Catfish (*C. gariepinus*) with Ovaprim introduction which was quite like our current study where the highest fertilization rate (83.66%), lowest as (45.66%) for the least hormone dose. Again, hatching rate of eggs were observed as 40.33±8.50, 80.0±3.60, 72.66±5.68 and 50.66±9.01 in respect to 0.25, 0.5, 1.0 and 1.50ml kg^{-1} BW hormone dose respectively (Figure 1). The highest hatching rate revealed 80.0±3.60 was quite lower that of Borah et al., (2020) found 92.49% in *M. pancalus* with Ovasis treatment. The T_2 treatment was found to be efficient in case of hatching and fertilization success.

Water quality profiles such as pH, temperature, and transparency of different treatments of *M. cavasius* ranged from 6.67-6.93, 29.33-30.33 °C, and 22.67-23.57 cm, respectively (Figure 2). Physico-chemical water parameters might play a significant role to improve fertilization rate and successful hatching. Current study findings denoted a good environment for successful induced breeding (Chand et al., 2011). In addition, optimum environmental conditions ameliorated the breeding performance in *Labeo bata* using Ovaprim and ovatide hormone (Behera et al., 2007), *M. pancalus* administrated with PG hormone (Alam et al., 2009) and in induced breeding of *A. testudineus* by using S-GnRHa (Rahman et al., 2021).

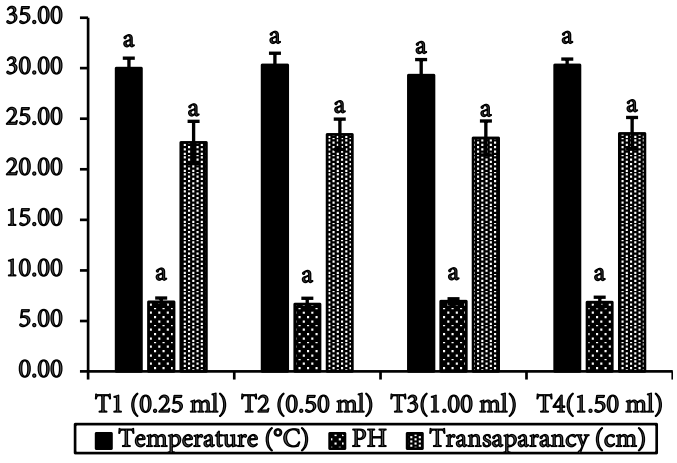

Figure 2. Physico-chemical water quality parameters of the breeding ponds

In our current study, the fertilized eggs of *M. cavasius* were strongly adhesive. Puvaneswari et al. (2009) reported that siluriform fishes showed adhesive forms of the eggs, for instance, fertilized eggs in *P. pangasius* are sticky and jellied in nature (Ferosekhan et al., 2015). Table 2 shows the embryonic development and interval time of observation in *M. cavasius*. The fertilized eggs (Figure 3b) of *M. cavasius* showed meroblastic cleavage in a first stage that divided the blastodisc into two blastomeres occurred within 20-25 min. after fertilization (Figure 3c). Nesa et al. (2017) obtained first cleavage in 00:21-2:09 h of post-fertilization to form 32-cell by administrating PG extract in *H. fossilis*, where the current study observed first cleavage up to 32-cell formation in 00:35-2:20 h. The morula phase was reached at 180-185 min. after fertilization (Figure 3i). At that stage, blastoderm spread over the yolk in and embryo became distinguished. Rahman et al. (2004) found the same stage of *M. cavasius,* however, quite earlier within 2:20 h after fertilization. Puvaneswari et al. (2009) and Nesa et al. (2017) also observed the same stage within 2:35 h of post-fertilization using Ovaprim and PG extract, respectively, in *H. fossilis*. Henceforth, blastula stage in the current study (3:30-4:00 h of post-fertilization) was illustrated as flattened and compacted blastodermic cell with occupied moderate surface (Figure 3j). Sarma et al., (2012) reported for *O. pabo* that blastula stage was observed in 3:30 h and one third of egg surface was coated by the blastoderm cells as well.

The gastrula phase arrived at 5:0-5:30 h after fertilization and traced through further expansion of the blastoderm taking around 60-70% surface (Figure 3k) and give rise to thread like germinal rings (Figure 3l). The "C" shape embryonic stage appeared at gastrula stage (Figure 3l). The head and tail regions become clearly prominent, and embryo appeared as encircled over the yolk sphere (Figure 3m). Ferosekhan et al. (2015) reported that the gastrula stage of *P. pangasius* appeared at 7:27 h, germinal ring sustained up to 8:00-9:00 h. This stage appeared at 9:00-10:00 h in *O. pabo* (Sarma et al., 2012) and at 11:00 h in *P. sutchi* (Islam, 2005). Twisting movement of *M. cavasius* embryo in the present study was observed through unfolded from encircled over the yolk sphere. Tail became free and the head left still adhere to yolk sac. The beating of tail become fast just preceding to hatching. Ferosekhan et al. (2015) reported that beating of tail was around 50-60 times per minute at 22:00-23:00 h post-fertilization. The larvae started hatching at 24:00 to 25:00 h. The tiny larvae were clearly distinguished with separated head, trunk, and tail region (Figure 3n). Hatching occurred at 24:00-26:00 h (25:27 ± 01:28 h) in *P. pangasius* (Ferosekhan et al., 2015), at 22 h in *R. rita* (Mollah et al., 2011), and at 26 h in *C. batrachus* (Das, 2002).

The larvae were 160.60±8.97 µm mm in measurement. The mouth of *M. cavasius* was not visible after hatching. After 2:30±3:30 h hatching, the fin fold was differentiated. A transparent thin blurred fin encircles over the caudal region and stretched up to the yolk sac (Figure 3q). This type of changes also identified in newly hatched larvae of *P. pangasius* by Ferosekhan et al. (2015), and in *P. sutchi* by Islam (2005).

Table 2. Observation of the embryonic development and interval time of *M. cavasius* (T_2 treatment group were considered for embryology study and three replicates were maintained in all cases)

Phase	Duration and size (mean ± SD)	Description	Figure No.3
Unfertilized Egg	99.17±4.29 µm	Opaque and whitish in color	(a)
Fertilized egg	85.58±5.87 µm	Transparent, adhesive, and watery in color	(b)
Cell division	35-40 min	Cleavage	
Two cells	20-25 min; 90.0±3.08 µm	Two cells over the yolk vesicle were identified.	(c)
Four cells	35-40 min; 85.76± 1.93µm	Four cells (blastomeres) were observed.	(d)
Eight cells	60-65 min; 98.82± 3.88µm	Eight cells were identified and looked like fingers.	(e)
Sixteen cells	90-100 min; 84.17±2.36 µm	Sixteen cells were observed (blastomeres were overlapping).	(f)
Thirty-two cell	120-140 min; 80.30±1.99 µm	Blastomeres were arranged in three layers with overlapping observation.	(g)
Multi-cell (64 and 128)	150-160 min; 82.94±2.08 µm	Cell proliferation numbers observed within the egg. Three layers were observed.	(h)
Morula	180-185 min.; 86.11±1.13 µm	Cleavage resulted into 64 and 128 cells. Look likes flowery.	(i)
Blastula	3.30-4.0 h; 84.70±2.11 µm	The blastomeres covered about 30-40% area and become compacted.	(j)
Gastrula	5-5.30 h; 90.0±0.76 µm	Blastoderm spread in both the side which covered about 60-70% area and thread line germinal ring appeared.	(k and l)
Head and tail bud formation	8.30-9.0 h; 77.64±1.93 µm	Head and tail visible	(m)
Just before hatching	23:30 h; 78.53±1.88 µm	Embryo encircled the whole yolk looking "c" shaped. Twisted movement of caudal region was started to unfold from the yolk sac.	(n)
Newly hatched larvae	24-25 h; 160.60±8.97 µm	Head, tail, and yolk sac were clearly identified as well as straight body observation.	(o and p)

Table 3. Observation of the larval development of *M. cavasius.* (T_2 treatment group were considered for embryology study and three replicates were maintained in all cases)

Age of larvae	Length (mm) of body and yolk sac	Characteristics	Figure No. 3
5 h	3.0±0.2 mm and 75 µm	Heart pumping identified. Yolk sac remained compact and eye yet identified.	q
10-11 h	3.3±0.1 mm and 66 µm	Eye clearly identified and melanophores were present around the yolk sac and brain region. A tubular heart appeared.	r
24 h	Yolk size 61.76±6.09 µm	Gradually reduced yolk from the yolk sac. Eye and anus identified. Blood circulation system fully functional.	s
4 days old	-	Yolk sac completely absorbed. Larvae freely swimming and started to eat natural food.	t

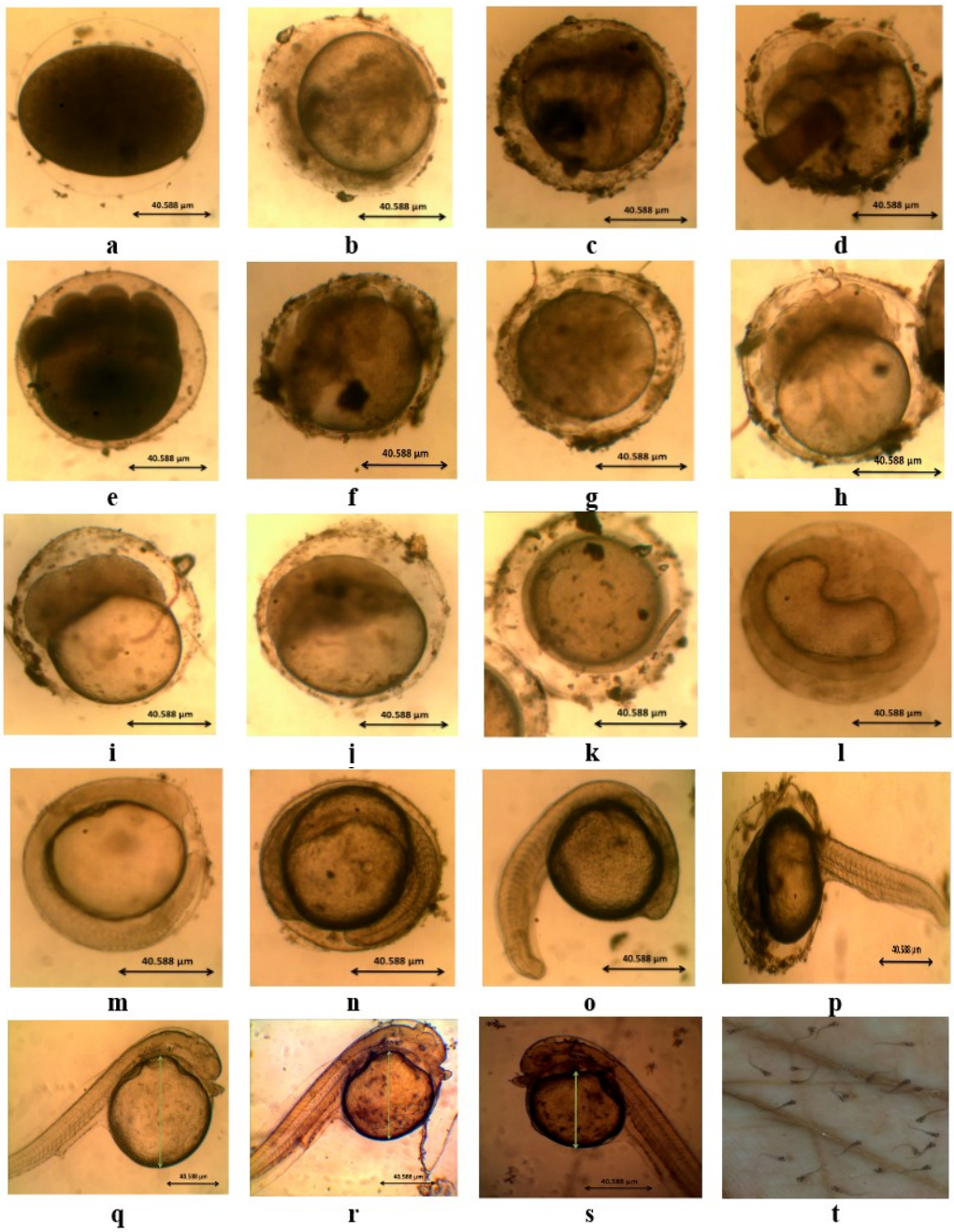

Figure 3. The Embryonic development of *M. cavasius*: (a) unfertilized egg; (b) fertilized egg; (c) two cell; (d) four cell; (e) eight cell; (f) sixteen cell; (g) thirty-two cell stage; (h) multi-cell (about 64); (i) multi-cell (about 128) stage; (j) morula stage; (k) blastula stage; (l) gastrula stage; (m) head and tail stage; (n) just before hatching; (o) newly hatched larva; (p) newly hatched larva (advanced stage); (q) Five hours after hatching; (r) ten hours after hatching; (s) twenty-four hours old larvae; (t) four days old larvae.

The position of the 5 h old larva's mouth and the eyes were non pigmented (Figure 3q). At that stage, a dorso-ventrally irregular fin appeared, and the larvae tried to start swimming. The swollen yolk gradually elongated when they were 10-11 h old, besides some melanophores appeared on the head region and around the yolk sac (Figure 3r). Afterwards, the barbell was partially observed. Rahman et al. (2004) reported that the barbell of *M. cavasius* was found in 6-12 h old larvae. The 24 h old larva with a reduced yolk sac appeared with dark pigmented anterior part of the head and prominent eyespot, meanwhile, two pairs of barbells were also noticed. The circulatory system was reported to be fully operating (Figure 3s). With the increase of age and time interval, gradual reduction of yolk sac was observed, which got completely absorbed at the end of third day (Figure 3t). The alimentary canal was also appeared at the age of three or four days and the larvae started having natural food items except yolk sac like as small zooplankton as feed. Rahman et al. (2004) reported that the yolk sac of *M. cavasius* was fully absorbed by 48 h old larvae. The round, dense yolk sac become reduced as the hatchling continued to grow in age and absorbed completely at the end of 72 hours of life before they take external foods (Islam, 2005; Ferosekhan et al., 2015).

Conclusion

The results of the current study suggests that a dose of 0.5 mg/kg body weight S-GnRHa application to females would be efficient for successful breeding induction in *M. cavasius* and better initiation of larval and embryonic growth. However, designing an experiment with a larger sample size is required for analyzing the further effects of this dose in aquafarms.

Compliance With Ethical Standards

Authors' Contributions:

This research includes MSc thesis work of first author. The chronology of author list reflects the contrition level of different author. However, the last author will be treated as team leader and principal supervisor as per institutional policy.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

This study was duly compiled with all sorts of regional, institutional and national animals ethics clearance.

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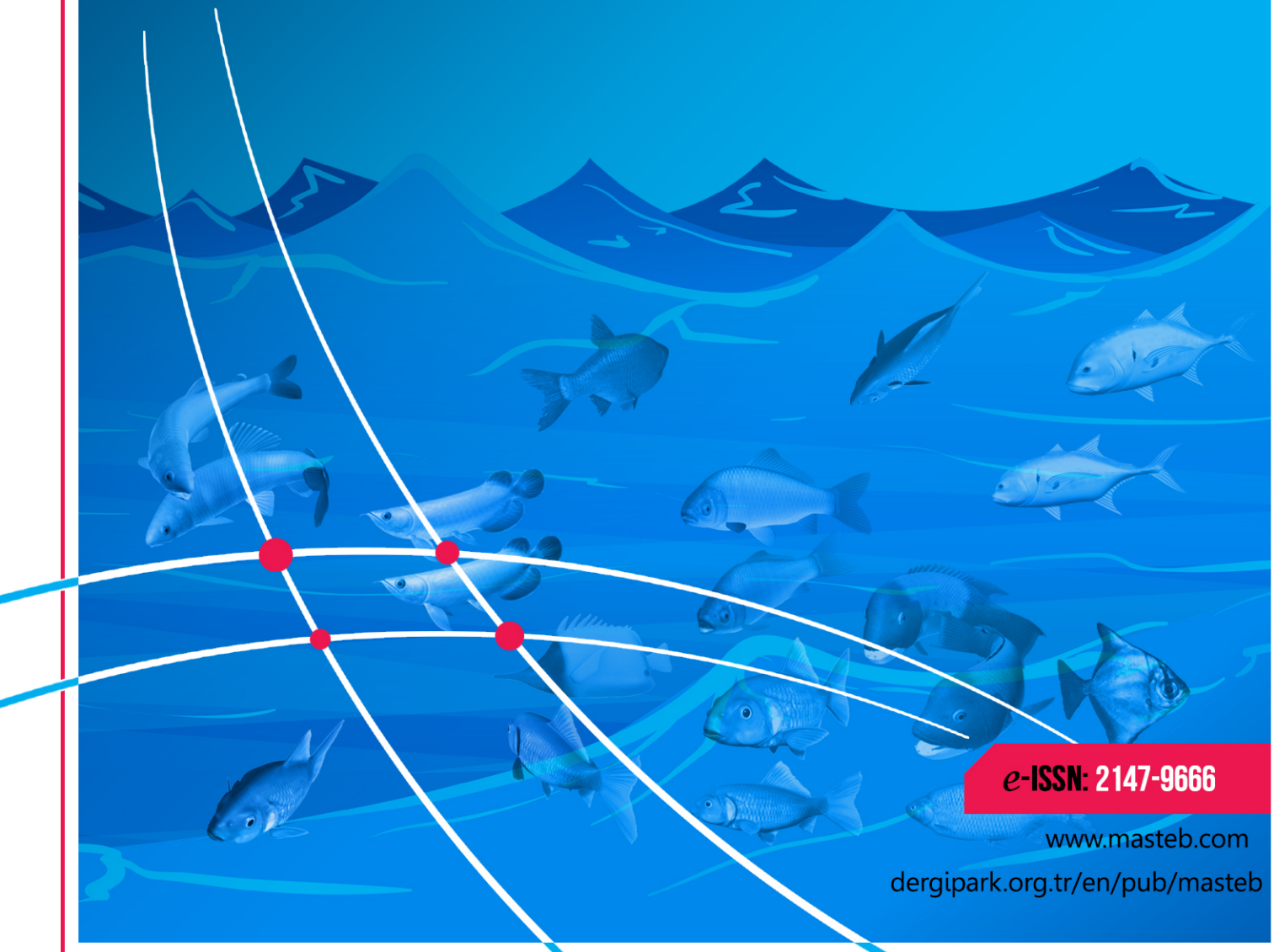

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